Effect of Elevated Water Temperature on The Reproductive Physiology of Female Atlantic Salmon (*Salmo salar*) Farmed in Tasmania

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for the Degree of
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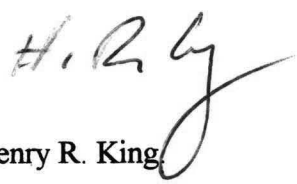
DECLARATION

Except as stated herein this thesis contains no material which has been accepted for the award of any higher degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgement is made in the text.



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ABSTRACT

The culture of Atlantic salmon (*Salmo salar*) in Tasmania, Australia is conducted at latitudes (~42°S) where natural water temperatures frequently approach the upper limits of thermal tolerance. Accordingly, experiments were conducted to characterise the reproductive development of Tasmanian female Atlantic salmon, to assess their temperature sensitivity and to examine the endocrine effects of maintenance at elevated temperatures. Under natural conditions, the development of Tasmanian female Atlantic salmon was broadly consistent with the recognised pattern of salmonid reproductive development. Between October and March, gonadosomatic index (GSI %) increased linearly from 0.5 to 17 %, oocyte diameter increased from 1.3 to 5.4 mm and oocyte volume increased 70-fold. Simultaneously, plasma estradiol (E_2) and testosterone (T) each increased exponentially (~3 to 20 ng.ml^{-1}).

During maturation (April – June), plasma E_2, T and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) displayed characteristic pre-ovulatory peaks (40, 50 and 75 ng.ml^{-1} respectively) in fish held at 6°C (equivalent to natural conditions). At 11°C, similar steroid peaks and associated ovulations were delayed (11-14 days). Luteinizing hormone releasing hormone analogue (LHRHa) treatment accelerated steroidogenesis and ovulation at 6 and 11°C. In contrast, at 16°C steroidogenesis was impaired and ovulation was inhibited irrespective of treatment.

Temperature reduction to 11°C restored 17,20βP production and ovulation in fish previously held at 16°C. Similarly, following temperature reduction, 17,20βP production and ovulation were accelerated in response to 17α-hydroxyprogesterone (17P) and/or LHRHa treatment. Egg fertilities were similar (84.5-94.0%) in all treatments at 11°C, whereas under temperature ramp-down, fertilities were reduced in controls (32.0%) and fish receiving 17P only (48.7%) relative to those receiving LHRHa (>80%). Although no ovulations occurred in fish held at 16°C throughout, significant 17,20βP production (up to 45 ng.ml^{-1}) was observed following 17P injection, indicating 20β-hydroxysteroid dehydrogenase (20β-HSD) activity.

During summer (January – February), maintenance at 22°C resulted in reduced E_2 and vitellogenin (Vtg) production (~1.8 ng.ml^{-1} and ~5 mg.ml^{-1} respectively) relative

to 14 and 18°C (>2.5 $ng.ml^{-1}$ E_2 and >10 $mg.ml^{-1}$ Vtg). In contrast, plasma cortisol levels tended to be elevated in fish held at 22°C (~ 44 - 103 $ng.ml^{-1)}$ relative to those in fish held at 14 and 18°C (~ 41 - 73 $ng.ml^{-1}$). Following autumn temperature reduction, oocyte diameter and egg survival were significantly reduced for fish held at 22°C (5.4 mm and ~42%) relative to lower temperatures (>5.65 mm and >84%).

Collectively, these studies demonstrate that reproductive development in Tasmanian female Atlantic salmon is highly temperature sensitive and that, in the absence of specific management measures, reproduction in Atlantic salmon cultured in Tasmania will be constrained by increases in temperature arising as a result of either global climate change, or efforts to phase-shift the timing of spawning.

Dedicated to the memory of

H.F. (Fred) Foster

6 August 1914 – 13 April 2000

" No Complaints "

ACKNOWLEDGEMENTS

The present studies were supported by Salmon Enterprises of Tasmania Pty. Ltd. (SALTAS), the Co-operative Research Centre for Aquaculture (Aquaculture CRC) and the University of Tasmania. Accordingly, I would like to express my gratitude towards the Board of Directors of SALTAS and in particular, the Chairman, Mr. Peter Morgan.

Experiments could not have been conducted without direct assistance from SALTAS staff members. Therefore, at the risk of missing someone, thanks must go to the following past and present employees; Mark, Eoin, Ange J., Ange P., Joey, Jody, Garry, Jo and Michele. Particular thanks must also go to SALTAS General Manager Mr. Graham Martin for his support, encouragement and friendship over the years that we have worked together as the "Management Team".

While production of this thesis represents a relatively early component of my development in academic terms, it also represents the completion of a journey from Fish Farm Manager to Scientist. I met a number of people along the way and their help must also be acknowledged. First, Pheroze Jungalwalla appointed me as a research officer with SALTAS and facilitated my migration to Australia from Scotland. Thereafter, in my role as research officer, I received a great deal of help and encouragement from members of the international scientific community. Liaison with John Thorpe, Gilles Boeuf, Ray Johnstone, Dick Saunders and Jim Duston lead me, via Jenny Specker, to Graham Young and, ultimately, Ned Pankhurst.

Clearly, in his supervisory role, Ned has had a significant influence on the present work and my academic development. More importantly however, he has become a great mate and I would like to take this opportunity to thank him for his advice, encouragement and for exposing me to his twisted sense of humour. Visits to the lab were accompanied by accommodation in the Pankhurst home. Therefore, thanks must also go to Ned's family – Tish, Sam and Mike – for their friendship and hospitality.

Data analysis always threatens to be a headache. Therefore , I cannot overstate the value of the advice and assistance provided by Assoc. Prof. Malcolm Haddon (Leader – Resource Modelling Section, TAFI Wild Fisheries Program) and Dr.

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Thanks to the family in Scotland (Mum, Dad, Nan, Duncan, Morag, Pete) for their love and support.

Finally, here in Tasmania, thanks to my son Kenny for sleeping through the night from a very early age and for not eating too much of my reference collection, and thanks to my wonderful wife Catriona – the reasons are too numerous and too complex to describe and they are the reasons I love you.

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"The introduction of the salmon rises almost above an experiment in acclimatisation. It is a great national work, whose value is perhaps second only to that of the introduction of the merino sheep to New South Wales by Colonel MacArthur. As an article of commerce alone, the value of the salmon to us is inestimable."

Melbourne *Argus* – April 1864

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 Atlantic salmon farming in Tasmania

1.1.i The development of salmon farming

The present day farming of salmonids largely stems from the freshwater pond farming of rainbow trout (*Oncorhynchus mykiss*) as it was practiced in Denmark at the end of the nineteenth century (Monahan, 1993; Tidemand-Johannessen, 1999). The Danish technology was adopted first in Norway where its success was limited due to the lower water temperatures and thus reduced growing season in that country. However, it was observed that the warmer coastal waters could also be employed and the first attempt at seawater rearing of rainbow trout was made in 1912 (Tidemand-Johannessen, 1999). Unfortunately, those early attempts failed and it was not until the 1960s that renewed efforts yielded significant advances. At that time, the economics of production also favoured the introduction of the more valuable Atlantic salmon (*Salmo salar*), and the development of reliable sea-cage technology lead to the establishment of a profitable industry. Subsequently, the technology and processes have been adopted around the world in countries such as Scotland, Ireland, Chile, Iceland, Canada, U.S.A. and Australia (Tidemand-Johannessen, 1999).

As a result of the above technological developments, production of farmed Atlantic salmon in Norway rose to approximately 370,000 metric tons in 1999 with world-wide production of the species reaching approximately 750,000 metric tons (Hansen et al., 2000). These high levels of production have been further facilitated by changes in management practices, in particular, utilisation of photoperiod manipulation techniques, which have served to reduce the seasonality of production (Hansen et al., 2000).

1.1.ii The development of Atlantic salmon farming in Tasmania

The commencement of cultivation of salmonids in Tasmania, Australia predates that in the majority of the better-known or recognised salmon farming regions of the world. In fact, the first successful introduction of brown trout (*Salmo trutta*) outside its natural range of Europe and Southwest Asia, occurred in 1864 on the Plenty River,

Tasmania (Laird, 1996). On 21 January 1864, the sailing clipper *Norfolk* sailed from London carrying approximately 100,000 Atlantic salmon ova collected from rivers in Scotland, England and Wales along with approximately 3,000 brown trout ova from three English rivers. The ova were placed, 400-500 at a time, in small perforated wooden boxes between layers of damp sphagnum moss and charcoal, and the boxes were packed under 30 tons of lake ice in an ice-house constructed on the vessel's lowest deck, amidships and midway between the stem and stern. After 84 days at sea, the *Norfolk* docked in Melbourne and the majority of the ice and boxes of ova were transhipped to Hobart aboard Her Majesty's War-sloop *SS Victoria*. The *Victoria* anchored in the Derwent river on 18 April and, following transfer up river by barge and land to a previously prepared site on the Plenty River, the ova were unpacked and laid-down in gravel beds on 21 April, 91 days after leaving London (Clements, 1988).

The brown trout were an immediate success, resulting in the successful acclimatisation of the species in Tasmania, and soon after, New Zealand. However, in spite of the fact that fry, parr and smolts appeared to thrive on a diet of powdered boiled liver then fly maggots, the ultimate fate of the Atlantic salmon following their release is less clear. It has been speculated that inappropriate spawning practices may have resulted in their loss through hybridisation with the brown trout. Alternatively, they may have performed poorly in competition with the trout or moved offshore in search of prey or more favourable environmental conditions, and in so doing, become lost in the Southern ocean (Clements, 1988). Regardless, in spite of repeated introductions between 1866 and 1934 and in keeping with the general rule that the likelihood of successful introduction is greater with non-anadromous forms than with anadromous forms (Laird, 1996), Atlantic salmon failed to establish a breeding population in Tasmania (Clements, 1988; Jungalwalla, 1991a).

Interest in the commercial cultivation of salmonids in Tasmania grew with the establishment of freshwater rainbow trout farms in the 1960s and 1970s and with small-scale trials of seawater culture of rainbow trout in the late 1970s and early 1980s (Jungalwalla, 1991a and b). At that time, the Tasmanian Fisheries Development Authority (TFDA), with the assistance of Norwegian advisors, formulated a plan for the establishment of a seawater salmonid farming industry in Tasmania. As a result of a 1968 ban on the importation into Australia of fresh salmonid products, potential

source stocks were restricted to previously established land-locked populations on the Australian mainland. In one of these populations, Atlantic salmon originating from the River Philip in Nova Scotia, Canada had been introduced to a government hatchery following importations of eyed ova in 1963, 1964 and 1965. Potential broodstock from this population underwent extensive health testing for 18 months prior to spawning and in 1984, 115,000 eyed ova were transferred to a quarantine culture facility operated by the TFDA. After 15 months of rearing under quarantine, 36,000 smolts were transferred to sea-cages in order to establish the first Tasmanian broodstock. Subsequent imports of 180,000 and 375,000 eyed ova from New South Wales in 1985 and 1986 respectively, supplied smolts to industry following a similar quarantine protocol. Thereafter, further imports of stock to Tasmania were banned as the 1984 year-class matured to yield the first cohort of Tasmanian progeny in 1987 (Jungalwalla, 1991a,b).

As these imports were underway, Salmon Enterprises of Tasmania Pty. Ltd. (SALTAS) was established under the auspices of the Salt-Water Salmonid Culture Act – 1985 of the Tasmanian parliament (Clements, 1988; Jungalwalla, 1991a). The company was to operate a hatchery and smolt production facility along with an experimental sea-farm and research station with the objectives of establishing broodstock, producing a minimum of 1 million smolts per annum, training staff for industry, and commissioning and conducting research directed at assisting industry development. Furthermore, in order to control the early developmental phase of the industry, the Act of Parliament also provided SALTAS with exclusivity of operation as an Atlantic salmon hatchery for a period of 10 years (Jungalwalla, 1991a).

Initial equity participation in the company was A$ 2.5 million of which 51% was provided by the Tasmanian State Government, 19% by the Norwegian joint venture partner, A/S Noraqua and 30% by private investors (Clements, 1988), with the entitlement to purchase smolts defined on a *pro rata* basis, although the Government share attracted only 1% of the total smolt production for broodstock and research purposes (Jungalwalla, 1991a). In addition to the *pro rata* allocation of smolt numbers, shareholders received, *pari passu*, a share of the differing sizes of smolts produced (Jungalwalla, 1991b).

Significantly, the company's research function was associated with a requirement (expressed in the company's Articles of Association) to deploy 25% of the gross revenue from smolt sales to finance a Research and Development programme. Thus, for the ten years 1985-1995, using an R&D management process that incorporated priority setting, funding, review and industry capture mechanisms, SALTAS acted as an industry organ acutely attuned to, and accurately reflecting the R&D needs of the Tasmanian Atlantic salmon farming industry.

1.1.iii The current status of Atlantic salmon farming in Tasmania

By mid 1987, 13 separate companies were farming Atlantic salmon on a total of 18 sites (mean size 5 ha) in the West and South of the State in Macquarie Harbour (Figure 1.1a), the Huon Estuary, Port Esperance and D'Entrecasteaux Channel areas (Figure 1.1c) and on the Tasman Peninsula (Figure 1.1d; Jungalwalla, 1991a). The first harvests by many of these companies occurred in the late 1980s at a time when world salmon prices were in decline with the result that by the mid 1990s, only 6 marine farming companies were in operation. However, following the cessation of SALTAS' monopoly on smolt production in 1995, 5 additional hatchery companies have commenced operations or transferred from rainbow trout to Atlantic salmon smolt production while a further 3 marine farming operations have been established in the West (Figure 1.1a) and North (Figure 1.1b) of the state.

In spite of these developments, SALTAS continues to dominate smolt production in Tasmania and, with production scheduled at approximately 3.5 million in 2000 and 2001, the company will provide in excess of 60% of the Tasmanian industry's seed-stock. The company now operates from two sites located on the Derwent and Florentine rivers in the state's central highlands and has recently redeveloped its egg and fry rearing facilities to incorporate 95% water recirculation systems which may also be employed for the ongrowing of fry to the parr and smolt stages (Anon, 1999).

In describing the culture of Atlantic salmon in Tasmania it is important to understand the progress of the different stages of the animal's life-cycle in relation to the seasons, and in particular, relative to the progress of those same phases in Northern hemisphere stocks. In the single Tasmanian stock of Atlantic salmon, gamete

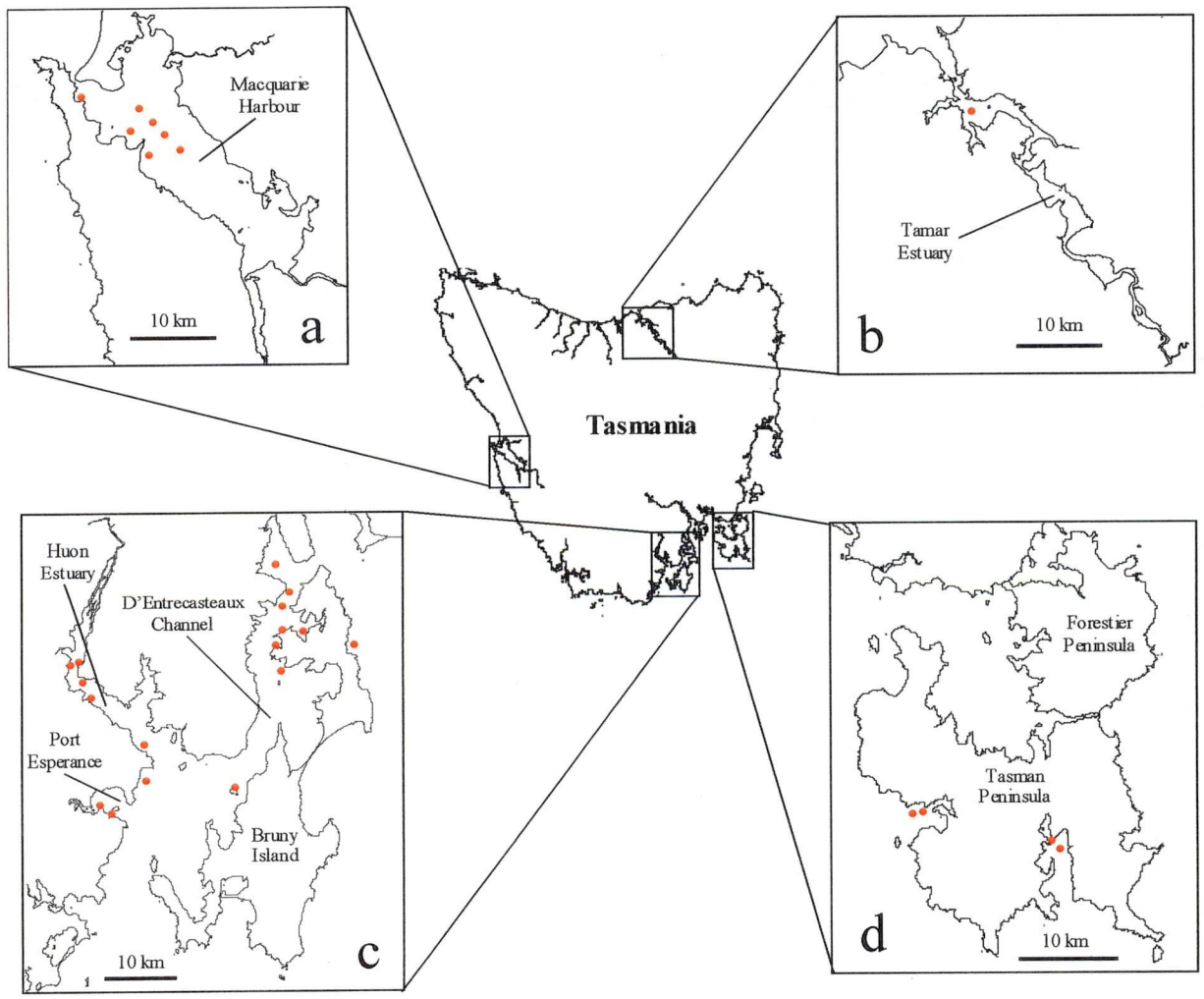


Figure 1.1 Location of Atlantic salmon farming activity in Western (a), Northern (b) and Southern (c & d) Tasmania.

maturation and release (spermiation, ovulation and spawning) occur over a relatively short period in mid to late autumn (late April – end May; Figure 1.2). In contrast, in the Northern hemisphere, where a variety of river stocks occur over a range of latitudes and climatic conditions, these processes can take place over an extended period during autumn and winter (October – January) (Willoughby, 1999). Egg incubation, particularly at typical Tasmanian culture temperatures (~ 8°C), also takes place over a relatively short time-frame. Fertilised ova develop to the eyed stage in early winter (June) and progress to hatch by mid winter (July), whereas these developments can occur in Northern hemisphere stocks from late autumn (October) to early spring (March) (Willoughby, 1999). Following consumption of the yolk-sac during the alevin stage, Tasmanian salmon fry typically first feed in the late winter (mid – late August) and grow to the parr stage (up to 5.0g mean weight) during spring (October – November). As before, the same processes in Northern hemisphere stocks can occur over an extended period between late winter (end February) and early to mid summer (June – July). In both the Northern and Southern hemispheres, the parr stage continues for approximately one year until smoltification, the physiological pre-adaptation which is critical to successful growth in seawater (Hoar, 1988), commences with the approach of spring. Thus in Northern stocks, the smolt migration or seawater transfers can take place from March until June (Willoughby, 1999), whereas in Tasmania, the recognised smolt window is represented by a period of only approximately 6 weeks between mid September and mid November (Jungalwalla, 1991a,b).

1.1.iv Sexual maturation of Tasmanian farmed Atlantic salmon

After transfer to seawater, a characteristic feature of the Tasmanian stock of Atlantic salmon is its propensity for early sexual maturation. In Northern hemisphere stocks a small proportion of fish exhibit sexual maturation after 1 winter of seawater residence (1SW) but most fish only mature after multiple winters at sea (MSW). In stark contrast, the Tasmanian stock has consistently exhibited greater than 95% 1SW maturation (Jungalwalla, 1991a,b). In Northern hemisphere stocks, such fish are referred to as "grilse" and, while they tend to be somewhat larger than their peers, they must be graded out of the population (grilse grade) and harvested at a relatively

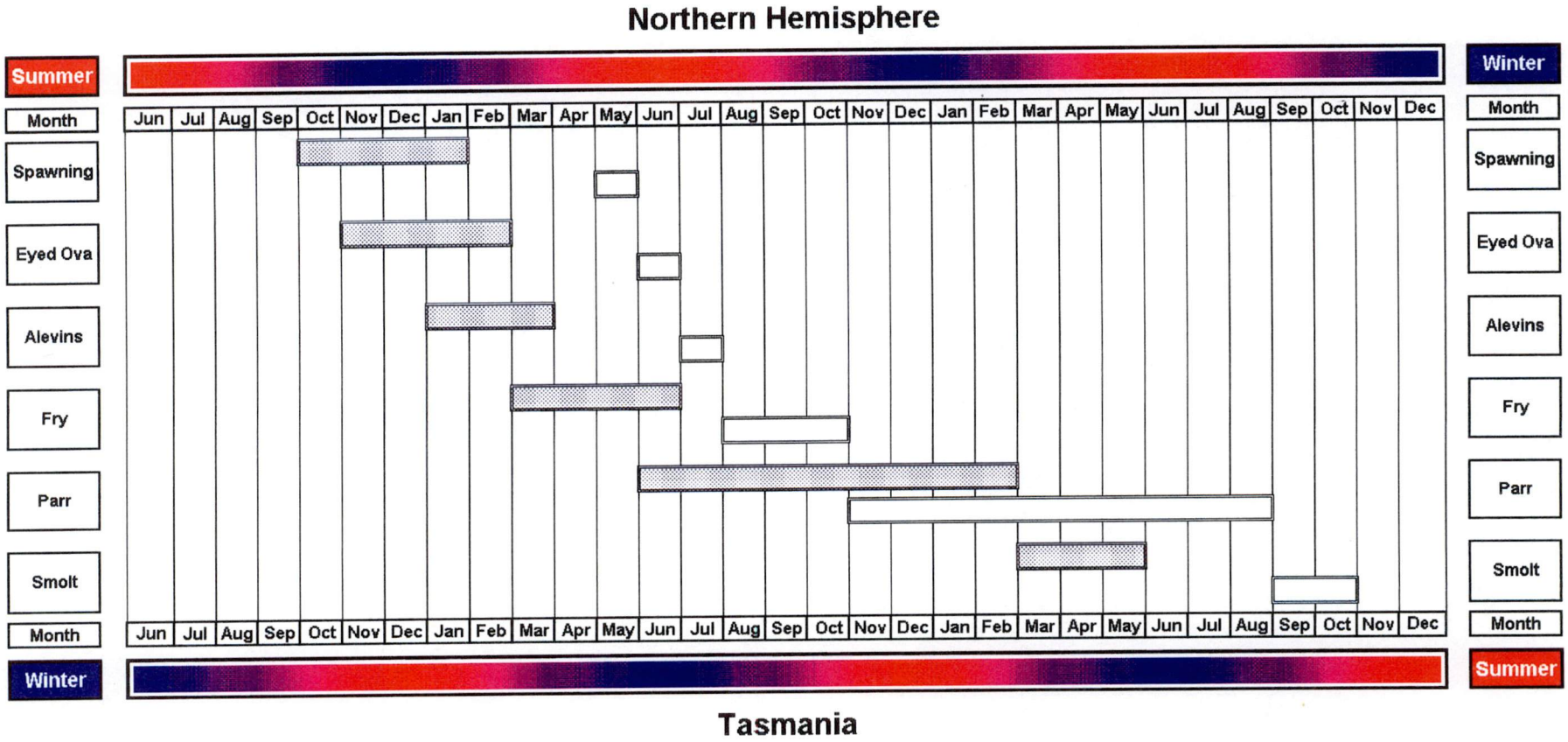


Figure 1.2 Timing of freshwater-phase life-history stages in Atlantic salmon farmed in the Northern Hemisphere and Tasmania.

small size (often less than 2.0 kg) prior to the development of undesirable secondary sexual characteristics (eg. development of sexual dimorphism, changes in skin colouration and reduction in flesh pigment levels) (Willoughby, 1999). In Tasmania, favourable growing conditions generally result in the majority of stock attaining mean weights of 3-5 kg prior to the onset of maturation (Jungalwalla, 1991a,b). Consequently, the phenomenon tends not to match the conventional "grilse problem". The phenomenon does, however, result in marked seasonality of production. Smolts transferred to sea during the recognised spring smolt window (Sept-Oct) take approximately 10-12 months to grow large enough for harvest and, thereafter, maturation and the onset of secondary sexual development render the fish unmarketable by the following March (Figure 1.3). Thus the viable harvest period is restricted to approximately 6-8 months of the year.

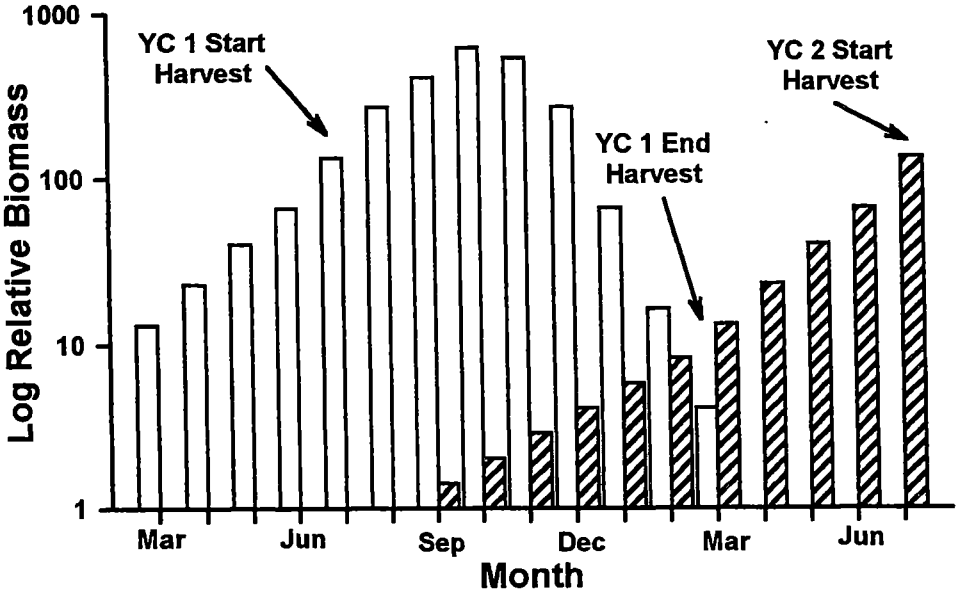


Figure 1.3 Biomass production of consecutive year-classes (YC) of Tasmanian Atlantic salmon following transfer as spring smolts. Arrows indicate typical 6-8 month harvest season. Clear bars represent YC 1 and hatched bars represent YC 2.

Control or avoidance of the grilsing phenomenon has been an important part of Northern hemisphere Atlantic salmon culture for many years (eg. Thorpe et al., 1990).

Significant efforts have been directed towards genetic selection to enhance the performance of "low grilse" stocks or family lines within stocks (Gjerde, 1993), and development of sterile stocks through ploidy manipulation (principally triploidy) has also been investigated (Johnstone et al., 1991). In Norway, strategic siting of operations along the country's extensive coastline has allowed some producers to take advantage of a range of temperature and photoperiod conditions to maintain year-round production (Rosten, KPMG Centre for Aquaculture and Fisheries, Trondheim, Norway, personal communication). More recently, increasing attention has been focussed on the application of photoperiod manipulation techniques to seawater cage-farming, to control the extent of sexual maturation in grow-out stock (see Oppedal et al., 1997; Taranger et al., 1998; Porter et al., 1999). However, as indicated earlier, the primary means of control or avoidance of grilsing, and thus maintenance of year-round production, is the use of photoperiod manipulation techniques during the freshwater phase of the fish's lifecycle in order to promote the development of 0+ smolts which are capable of transfer to seawater independent of season (Hansen et al., 2000). Furthermore, the process is particularly successful when combined with judicious size grading and harvest of the fastest growing (ie. those most likely to mature) post-smolts (Rosten, personal communication).

Similar approaches to the grilsing phenomenon have been examined or employed in Tasmania. However, selective breeding for low grilse lines was abandoned at an early stage in response to the acknowledged magnitude of the selection gradient. With grilse maturation rates exceeding 95%, it rapidly became apparent that significant improvements in the MSW maturation of the stock would take many generations to achieve. Moreover, in view of the relatively small genetic pools used to create first, the Australian mainland stock and second, the Tasmanian stock, the focus of the industry's breeding activities (bearing in mind the absence of molecular genetic tools at that time) became the retention of genetic diversity rather than the development of potentially highly inbred lines of fish with high-level expression of particular phenotypes (Ward et al., 1994; Reilly et al., 1999). As a result, considerably greater effort was directed towards the development of sterile stocks via the production of all-female triploids. In fact, in the late 1980s and early 1990s, it was envisioned that approximately 30% of all Tasmanian smolts would be triploid (Jungalwalla, 1991b).

At that time, research was conducted on the production of all-female stocks by the "indirect" method of feminisation where, following androgen treatment, genotypic females (XX) develop as phenotypic males and produce sperm which is used to fertilise eggs from untreated female fish, resulting in all female offspring (Hunter and Donaldson, 1983). Simultaneously, the induction of triploidy through the use of high pressure treatment to promote second polar body retention in ova soon after fertilisation (Benfey et al., 1988) was investigated. While this research was highly successful and the resulting fish performed well during the freshwater phase of production, problems were encountered early in the seawater phase. Most notably, high incidences of a jaw deformity were observed in rapidly growing post-smolts (Jungalwalla, 1991b). In appearance, the deformity resembled one reported by Bruno (1990) and which is often referred to as "trumpet mouth" by Northern hemisphere growers (Figure 1.4). In addition, the fish tended to be less robust and more susceptible to crowding and handling stresses than their diploid peers (Sadler et al.,

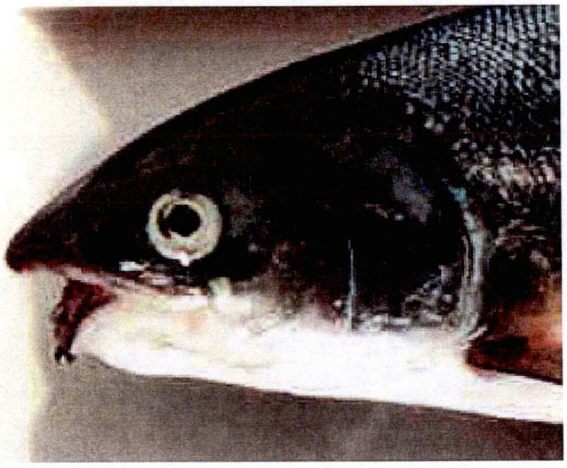


Figure 1.4 Female triploid Atlantic salmon displaying typical "Trumpet Mouth" deformity of the lower jaw.

2000a,b). More recently, it has also become apparent that, in addition to the obvious jaw deformity, all-female triploid stock exhibit branchial and opercular deformities at very high incidences (Sadler, 2000). As a result, while Jungalwalla (1991b) expressed confidence that the marine phase difficulties associated with rearing all-female

triploids would be transient, the popularity of these fish with the marine grow-out sector of the Tasmanian salmon farming industry has waned. Nevertheless, the 1SW maturation problem peculiar to Tasmania is so significant that all-female triploid smolts continue to be produced, albeit in lower numbers than was at first envisaged (typically 5-7%), in order to supply the market for a restricted part of the year (ie. the month of April).

Tasmanian marine farmers have also commenced experimentation with photoperiod manipulation of sea-caged stock but these efforts might, at best, be described as piecemeal and as a consequence, the results to date have been inconclusive. It is therefore, not surprising that, as in the Northern hemisphere, photoperiod manipulation of parr and production of 0+ smolts represents the primary means of achieving year-round harvests in Tasmania. That said, significant differences from standard Northern hemisphere practice exist. First, early in the development of photoperiod manipulation protocols, it was observed that growing conditions were sufficiently favourable to promote precocious maturation (jacking) in the male component of mixed-sex 0+ smolt populations after only 6 months of seawater residence (King, unpublished observations). As a result, the earliest transferred batches of 0+ smolts for commercial culture are now produced from all-female stocks. Second, whereas 0+ smolt numbers in countries such as Norway may represent up to approximately 30% of production (Hansen et al., 2000), the magnitude of the 1SW maturation problem in Tasmania is such that up to approximately 70% of production comes from stock which are transferred to seawater as 0+ smolts.

In addition to 0+ smolts, those Tasmanian salmon farmers operating in Macquarie Harbour (Figure 1.1a) and the upper reaches of the Huon Estuary (Figure 1.1c) are able to take advantage of reduced salinity conditions that result from large freshwater inflows to facilitate the mid to late winter transfer of large parr or pre-smolts. These fish derive some growth benefit from reduced stocking densities and intermediate water temperatures resulting from the mixing of cold freshwater (~ 3-4°C) and relatively warm seawater (~ 8-10°C).

As a result of the above processes and developments, Tasmanian farmed Atlantic salmon production totalled approximately 10,000 tonnes for the 1999 calendar year

(ISFA, 2000) and, while this figure represents less than 1.5% of world production, the significance of the industry to a small island state, which is heavily reliant on its primary production, cannot be overstated. Against this background, and in spite of ongoing difficulties associated with the control of cage net biofouling (Hodson et al., 2000) and management of environmental impacts and a significant parasite infestation – amoebic gill disease (AGD) (Roubal et al., 1989) – Tasmanian farmed Atlantic salmon production is predicted to double over a 3-5 year timescale In this context, the industry's 1SW maturation problem will continue to be an important factor constraining production efficiency. Although year-round production has been achieved, significant seasonal variation in production volume remains and as a result, further efforts to extend 0+ smolt production will entail manipulation of broodstock in order to advance the timing of spawning and all subsequent phases of the freshwater production process up to and including seawater transfer.

1.2 The Tasmanian salmon farming environment

1.2.i The Tasmanian climate

Tasmania is located to the south of the Australian continent where it is bisected by the 42nd parallel. At this latitude, a significant feature of the State's location is the fact that it is subject to persistent strong to gale-force westerly winds which occur at latitudes below 40°S and are known as "the roaring forties". These persistent onshore winds result in Tasmania's climate being markedly influenced by the waters of the Southern Ocean and, further to the west, the Indian Ocean. In addition, the presence of the Pacific Ocean to the east of the State means that Tasmania is influenced by three oceans (Cresswell, 2000) and as a consequence, the State's climate might best be described as maritime temperate. On the whole, daytime air temperatures are cool to mild with summer temperatures averaging approximately 18-24°C while average winter temperatures are approximately 9-15°C. Tasmania is Australia's most mountainous State but, while snow falls regularly above 500m, it is rare for temperatures to remain below freezing for more than 1-2 days at a time, even at elevations up to 1000m. Annual rainfall is moderate to high and the general range is approximately 600-800mm. However, west facing mountain slopes may receive as much as 2600mm while areas of south eastern Tasmania are severely rain-shadowed

and may receive as little as 500mm (Australian Bureau of Meteorology, http://www.bom.gov.au/).

1.2.ii Typical freshwater temperatures

As a result of the above air temperature and rainfall patterns, and in some areas the existence of large hydro-electric water storage lakes, many of Tasmania's larger rivers possess sufficient consistency of flow coupled with water temperature regimes which would be regarded as favourable for efficient salmonid growth (Wedemeyer, 1996). For example, water temperature data collected at SALTAS Freshwater Operations over the ten years 1986-1996 indicate a range in the ten-year monthly average temperature from approximately 5.7°C in winter to 15.6°C in summer with annual monthly average temperatures ranging 0.5-3.0°C above or below the ten-year average for that month (Figure 1.5). Values for mean monthly temperature are most consistent in late winter (August) and most variable in late spring and summer (November to February).

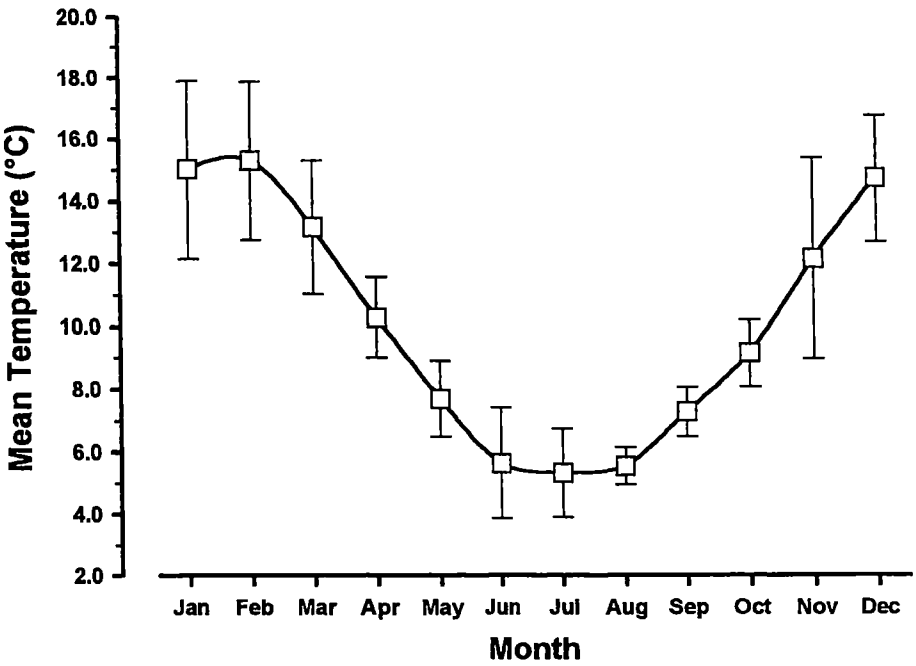
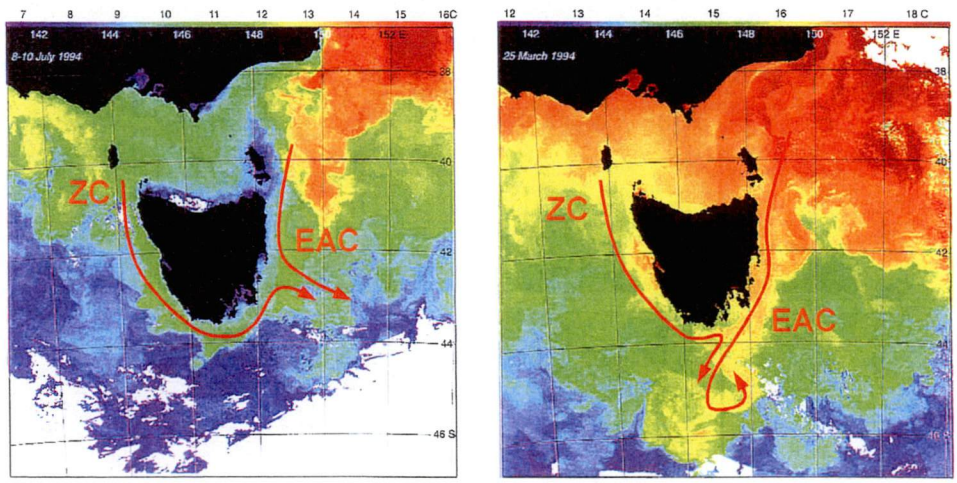


Figure 1.5 Mean water temperatures recorded at SALTAS Freshwater Operations (1986-1996). Bars represent range of mean temperature for each month.

1.2.iii Typical seawater temperatures

The influence of the Pacific, Indian and Southern oceans on the coastal waters of Tasmania are mediated by two currents – the Zeehan Current and the East Australian Current (Cresswell, 2000). In winter, the Zeehan current carries relatively warm water (~12°C) southward down the western Tasmanian continental shelf, around to eastern Tasmania, and then northward to approximately 43°S where it encounters the East Australian Current (Figure 1.6a). In contrast, in summer (Figure 1.6b), the Zeehan Current only extends as far as southern Tasmania before it is entrained into the East Australian Current which carries warm water (~17°C) southward down the eastern Tasmanian continental shelf, and overshoots approximately 200 km beyond the island's southernmost point. As a consequence of these current flows, coastal water temperatures in Tasmania's salmon farming areas typically range between 8 and 18°C (Jungalwalla, 1991a) and, consistent with the island's typical freshwater temperature regime, also favour efficient growth. Winter temperature minima are modified by cool freshwater inflows while in summer, temperature maxima can be strongly influenced by solar heating effects. For example, relatively warm waters can accumulate in Macquarie Harbour in spite of widespread cold up-welling on the adjacent northwest coast (Figure 1.7).



a **b**

Figure 1.6 Winter (a) and summer (b) sea surface temperature images for the Tasmanian region. Arrows indicate the movement of the Zeehan Current (ZC) off western Tasmania and its interaction with the East Australian Current (EAC). Adapted from Cresswell (2000).

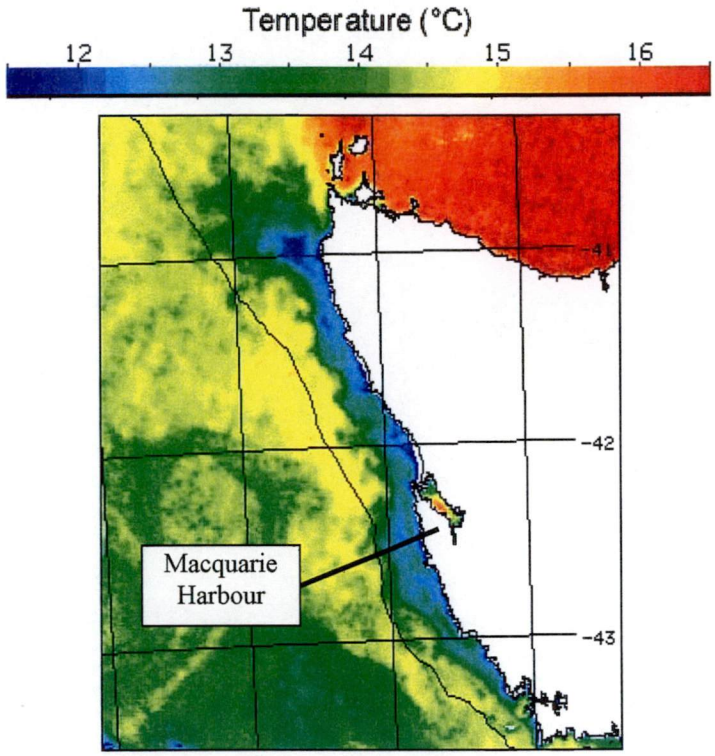


Figure 1.7 Optimally interpolated sea-surface temperature mosaic from orbits 24663-24665 of the NOAA12 satellite, 12 February 1996. Cold water up-welling along the northwest coast of Tasmania (blue) contrasts with warm water on the north coast and in Macquarie Harbour (red). Copyright 1996, CSIRO Marine Resources.

1.2.iv Climate variability in Tasmania

From the above descriptions, it is tempting to consider Tasmania's climate as relatively benign. However, Tasmania also shares continental Australia's marked climate variability. With the exception of Antarctica, Australia has the lowest and most variable rainfall of any continent and much of that variability is connected with the atmospheric phenomenon termed the Southern Oscillation and the related oceanic phenomenon known as El Niño which represents an oceanic warming event in the eastern Pacific (first recognised and named "the Christ child" by South American fishermen) and which occurs every two to seven years (Karoly, 1998). The Southern Oscillation represents the tendency of atmospheric pressure in the Pacific to increase while the pressure in the region of the Indian Ocean decreases, and is measured by gauging the sea-level pressure in Tahiti and in Darwin, Australia and using the

difference to calculate the Tahiti-Darwin Index or Southern Oscillation Index (SOI) (Allan et al., 1996). While El Niño and fluctuations in the SOI can occur separately, the two phenomena are typically coupled such that it is more appropriate to refer to El Niño-Southern Oscillation (ENSO) events, where high negative values of the SOI are associated with an El Niño or "warm event" (Allan et al., 1996). Under these conditions, atmospheric pressure is elevated in the Australian/Asian region while the seas around northern Australia cool and the easterly "trade winds" slacken and feed less moisture into the Australian/Asian region. As a result, there is a reduced likelihood of rain in eastern Australia leaving the region prone to extreme drought and exposed to the risk of bush-fire (Allan et al., 1996). Importantly, the high pressure conditions and associated reduction in cloud cover also increase the solar heating effect in south eastern Australia (Australian Bureau of Meteorology, 1998). As a result, inland freshwaters and inshore seawaters can experience significant warming in addition to that which normally occurs as average maximum temperature and the frequency of hot days increases (Australian Bureau of Meteorology, 1998).

There are no data to suggest any direct linkage between ENSO events and variations in the East Australian current. However, whether as a direct result of alterations in oceanic circulation or as a result of increased solar heating, summer water temperatures exceeding seasonal norms were observed prior to, during and following the El Niño year 1998 (Figure 1.8a-c). Thus, in recent years, prolonged elevation of the water temperatures in Tasmania's salmon farming areas has been common.

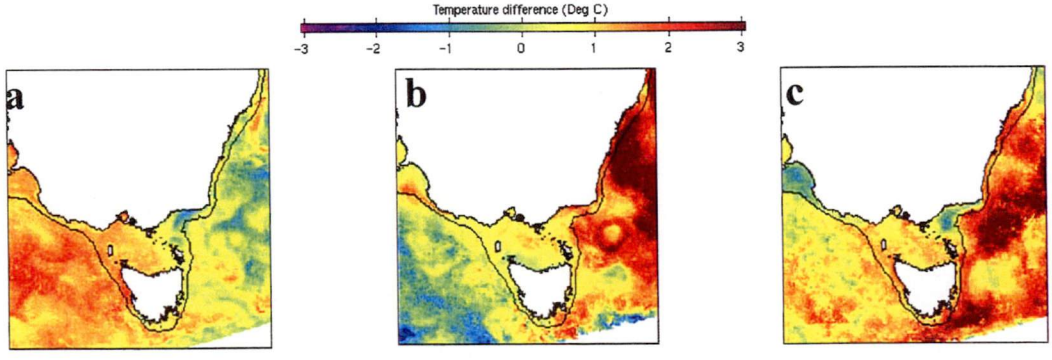


Figure 1.8 Optimally interpolated sea-surface temperature anomaly for a) 14 February 1997, b) 09 February 1998 and c) 14 February 1999. Areas coloured red indicate water at temperatures up to 3°C above seasonal average. Copyright 1999, CSIRO Marine Resources.

In inshore seawaters, maximum temperatures greater than 22°C have been recorded while temperatures exceeding 19-20°C may persist for some weeks (Figure 1.9).

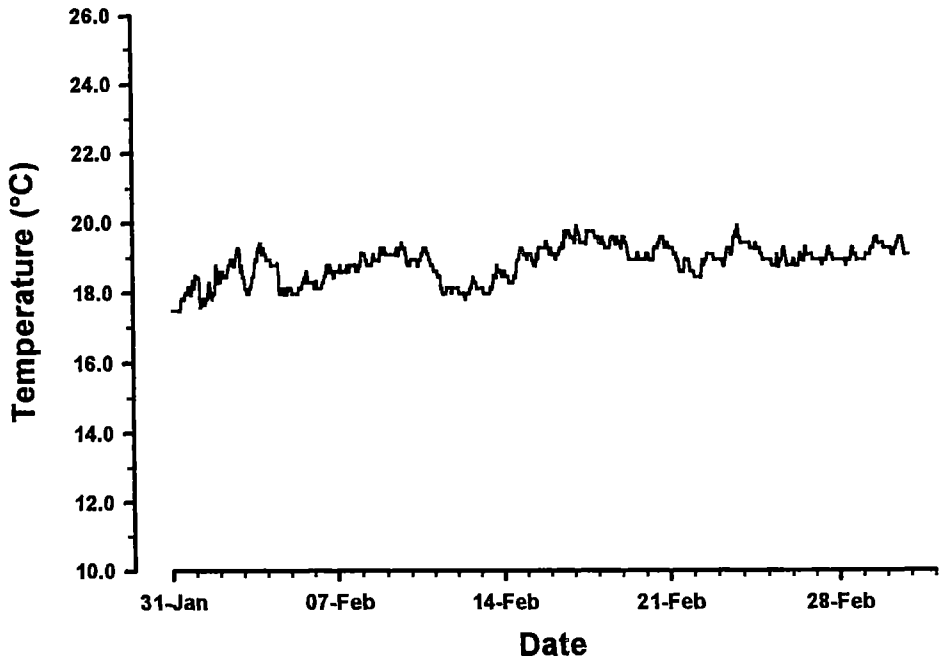


Figure 1.9 Sea-surface temperature recorded at Northwest Bay (SE Tasmania) during February 2000 (Data courtesy of Aquatas Pty. Ltd).

Similarly, data collected at SALTAS Freshwater Operations during recent summers indicate that river temperatures may exceed 25°C on occasion while daily temperature maxima of 20-22°C are common (Figure 1.10). Importantly, unlike freshwaters which tend to cool overnight (Figure 1.10), inshore seawaters tend to display less diurnal variability, remaining relatively warm throughout the day and night (Figure 1.9).

1.3 Salmonid thermal habitat preferences

In discussing the culture of Atlantic salmon in general and in particular, the culture of the species in regions outside of its natural range, such as Tasmania, it is worthwhile to consider the animal's natural habitat preferences. Specifically with regard to thermal habitat, there is growing evidence for a significant correlation between changes in sea surface temperature and changes in the growth, sea age at maturity and survival of salmon (Hutchinson and Mills, 2000).

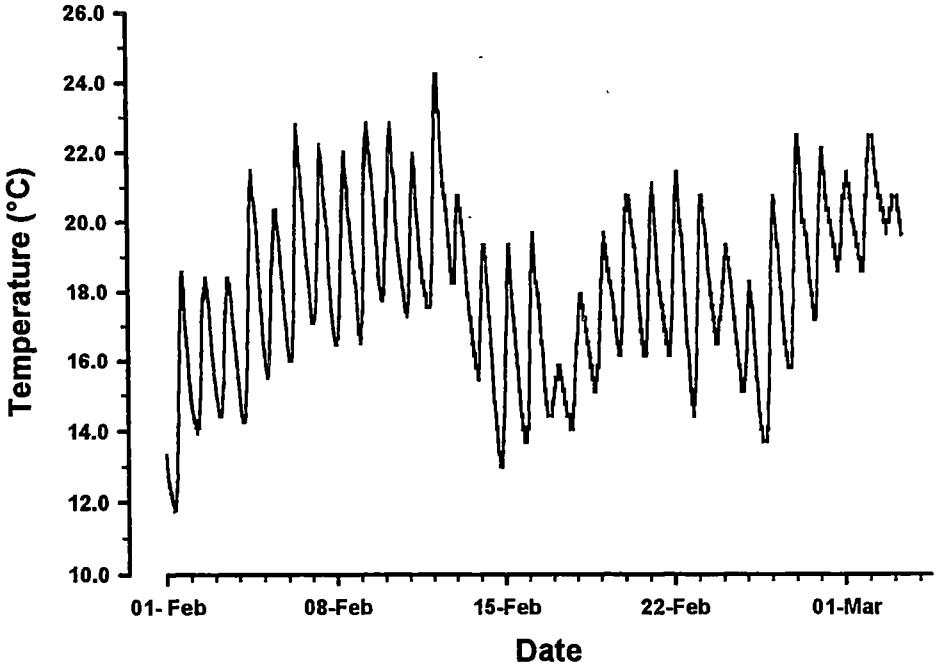


Figure 1.10 River Derwent water temperature recorded at Saltas Freshwater Operations (Wayatinah, Tasmania) during February 1999.

Near shore sea surface temperatures have been suggested to influence survival of both maturing salmon and post-smolts as they migrate to and from rivers (Reddin et al., 2000). For Atlantic salmon of North American origin, a temperature-based ocean habitat index has been correlated with pre-fishery abundance in potential two-sea-winter fish while the spring abundance of European stocks has been positively correlated with the area of ocean enclosed by the 7 and 13°C isotherms (Dickson and Turrell, 2000). In this context, the relevance of sea surface temperature as an oceanic property which might influence Atlantic salmon distribution is confirmed by recent observations which demonstrate that post-smolt and adult fish spend much of their lives at depths less than 10m (reviewed by Dickson and Turrell, 2000). Similar observations have been made for Pacific salmon (*Oncorhynchus* spp.) (reviewed by Welch et al., 1995). Thus, in the western North Atlantic, catch rates of adult Atlantic salmon decrease rapidly at sea surface temperatures below 4°C and above 8°C (Reddin et al., 2000), whereas highest catch rates occur in areas such as the central Labrador Sea and seaward of the West Greenland Current where water temperatures range between 5-8°C and 4-7°C respectively. Welch et al., (1995), using direct

observations of fish distributions, demonstrated that the locations of particular temperatures restricted the movements of four Pacific salmon species. Catch rates exhibited a sharp step-function response to sea surface temperature such that the critical upper temperatures defining the southern movement for chum (*O. keta*) and pink (*O. gorbuscha*) salmon, for coho (*O. kisutch*) salmon and for sockeye (*O. nerka*) salmon were 10.4, 9.4 and 8.9°C respectively. More recently, observations in the north-east Atlantic have indicated further restriction in the thermal habitat of Atlantic salmon of European origin. Emigrating post-smolts were observed to remain in waters between 8 and 10°C during their northward migration to feeding areas and the return rate of 1SW fish to both a Scottish (North Esk) and a Norwegian (Figgio) index river correlated with the size of the habitat area defined by the 8 and 10°C isotherms (Friedland et al., 1998). Interestingly, the dominant process affecting North Atlantic sea surface temperatures, and thus wild Atlantic salmon thermal habitat, is the North Atlantic Oscillation (NAO), the northern equivalent of the Southern Oscillation (Dickson and Turrell, 2000).

The above observations and correlations between fish distribution and water temperatures probably reflect the fact that fish tend to remain associated with oceanic features such as fronts, gyres and boundary currents for reasons of transport or access to prey. Moreover, Welch et al., (1995) speculate that salmon select lower temperature habitats in order to optimise growth under conditions where food may be limiting. However, regardless of the ecological and/or physiological basis for salmonid thermal habitat selection, it is clear that such choices are likely to be a direct consequence of evolutionary selection pressures (Welch et al., 1995). Therefore, it is not unreasonable to presume that many other aspects of the animal's physiology have also evolved to function optimally under the thermal conditions reflected in the preferred thermal range of wild populations. Thus, while salmonids might display optimal growth at approximately 16°C under conditions where food is not limiting, the animals are termed *stenothermal* in recognition of their narrow thermal tolerance range and it is generally acknowledged that optimum temperatures for processes such as spawning, egg incubation and smoltification are lower than those for growth (Wedemeyer, 1996).

Against this background, it is noteworthy that Tasmanian water temperatures can exceed even optimum growth temperatures for prolonged periods, frequently exceed 20°C and regularly peak above the recognised upper threshold lethal temperature range (22.0-23.5°C) for the species (Barton, 1996).

1.4 Temperature Implications for Reproduction

1.4.i Temperature effects on salmonid reproductive development

Temperature is known to affect reproductive development and the timing of spawning in all species including salmonids (reviewed by Van Der Kraak and Pankhurst, 1997). Low temperatures have been demonstrated to slow the processes of vitellogenesis and oocyte growth in salmonids and at very low temperatures final oocyte maturation and ovulation can be inhibited (reviewed by Scott, 1990). Elevated temperatures can also inhibit maturational processes. Gillet (1991) observed inhibition of ovulation at 11°C and a delay in ovulation at 8°C in Arctic charr (*Salvelinus alpinus*) whereas ovulation occurred as normal at 5°C. Similarly, Taranger and Hansen (1993) reported partial inhibition of ovulation in Atlantic salmon held at 13-14°C and Pankhurst et al., (1996) observed a similar response in rainbow trout maintained at 15 and 18°C. In addition, higher temperatures can adversely affect fecundity and gamete quality and fertility (Billard, 1985; Gillet 1991; Pankhurst et al., 1996; Pankhurst and Thomas, 1998).

There is little evidence that temperature acts as a proximate cue influencing reproduction in salmonids (Bromage et al., 1992, 1993) and seasonal phasing is almost totally dependent on photoperiod (Bromage et al., 1993). Because of this, it is probable that the reported effects of temperature on reproductive development and the timing of spawning are the result of effects on the endocrine system (Van Der Kraak and Pankhurst, 1997) including organs such as the ovary (Bromage et al., 1992). In this regard, there is currently relatively little information on the effects of temperature on the endocrine processes that control reproduction (Van Der Kraak and Pankhurst, 1997). Pankhurst et al., (1996) demonstrated that altered temperature regimes did not affect gonadal maturation in rainbow trout but did interfere with final oocyte maturation and ovulation. Further work showed that elevated temperatures delayed final maturation but that the endocrine machinery remained intact and could

be switched on later by treatment with exogenous hormones (Pankhurst and Thomas, 1998). There is also little information on the specific interaction of temperature with photoperiod (photoperiod manipulation has generally been examined in isolation). In a single study, Taranger et al., (2000) demonstrated that the effectiveness of photoperiod regimes designed to advance ovulation in Atlantic salmon, could be enhanced by reducing holding temperature. This, together with the characterisation of the endocrine mechanisms provided by Pankhurst and co-workers and the observations of Gillet (1991) and Taranger and Hansen (1993), indicates that elevated water temperature will be a constraining factor in any attempt to achieve a forward phase-shift in reproduction in Tasmanian stocks of Atlantic salmon (Figure 1.11).

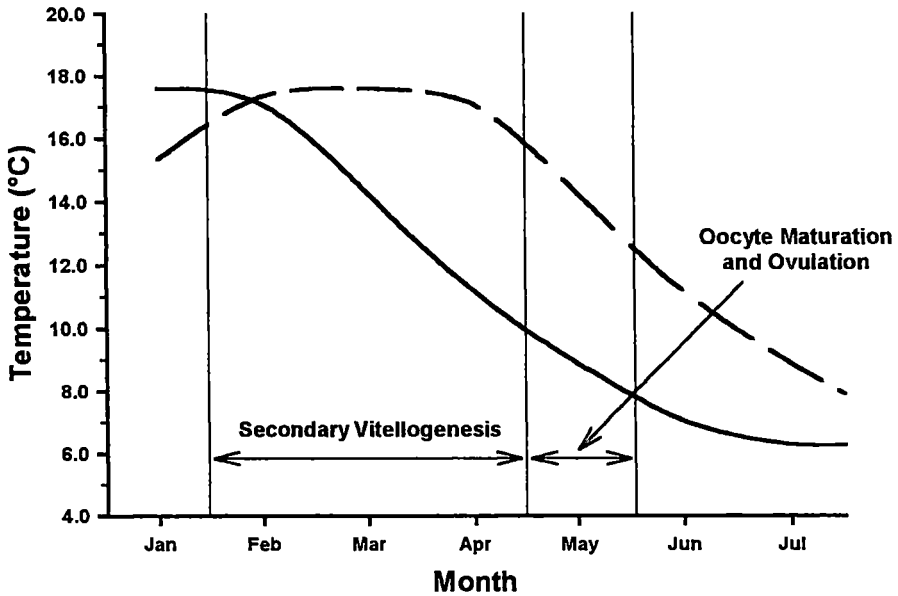


Figure 1.11 Typical water temperatures experienced during the final stages of reproductive development by maturing female Atlantic salmon in Tasmania (solid line) and the likely temperature profile in the event of a 2-month phase advance in the timing of ovulation (broken line).

An additional element of significance is added by the current concerns about directional changes in global climate and the fact that Atlantic salmon in Tasmania are grown at the upper limits of their normal thermal range. The possible effects of global warming on reproductive processes in fish have been reviewed (Van Der Kraak and Pankhurst, 1997) and it is clear that the information base upon which to make sensible

predictions about the effects of temperature change, in anything other than very general terms, is inadequate.

1.4.ii Reproductive development in female salmonids

It is important to note that there are significant sex-related differences in the response of salmonid reproductive processes to elevated temperature. For example, Billard (1985) reported 13°C as the upper temperature limit for salmonid ovulation whereas the fertilising capacity of rainbow trout sperm was unaffected by maintenance at 15 and 18°C relative to 10°C. Similarly, Duncan et al., (2000) showed that male Atlantic salmon exhibited a more marked response to advanced photoperiod than females. Spermiation and ovulation were advanced by up to 8 and 3 weeks respectively, and spermiation occurred when temperatures were as high as 17°C.

Reproductive development of female salmonids, generally takes place over a period of two or more years prior to ovulation, with vitellogenesis, the phase of rapid oocyte growth, occurring during the latter months of the cycle (Billard, 1985; Bromage and Cumaranatunga, 1988; Tyler et al., 1990). Throughout the process, environmental cues are perceived and transduced by the brain into neuroendocrine signals. Most importantly, gonadotropin releasing hormone (GnRH) is secreted by neurones of the hypothalamus and in turn stimulates the secretion of gonadotropins (GtHs) by the pituitary (reviewed by Peter and Yu, 1997). During vitellogenesis, GtH-I stimulates the 17β-estradiol (E_2)-mediated hepatic synthesis and follicular uptake of the glycolipophosphoprotein vitellogenin (reviewed by Swanson, 1991), and increasingly elevated plasma levels of E_2 and its precursor testosterone (T) are observed as vitellogenesis progresses (reviewed by Fostier et al., 1983). Later, during the final stages of oocyte development and prior to final oocyte maturation and ovulation, E_2 levels decline and T levels display a transitory peak associated with the fall in aromatization to E_2, followed by a decline (Figure 1.12; Fostier et al., 1983). Simultaneously, plasma levels of the progestin 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) increase sharply to high levels under stimulation by GtH-II (Figure 1.12; reviewed by Swanson, 1991). In turn, 17,20βP acts as the maturation-inducing hormone, stimulating the processes of final oocyte maturation (FOM) and ultimately, ovulation via the production of a maturation- or metaphase-promoting factor (MPF)

(reviewed by Nagahama, 2000). At this stage, dopamine (DA) can also have an important control function as it inhibits both GnRH and GtH-II secretion (reviewed by Peter and Yu, 1997). However, in salmonids, the regulatory role of DA may not be as important as it is in other teleost groups such as cyprinids (Van Der Kraak et al., 1986).

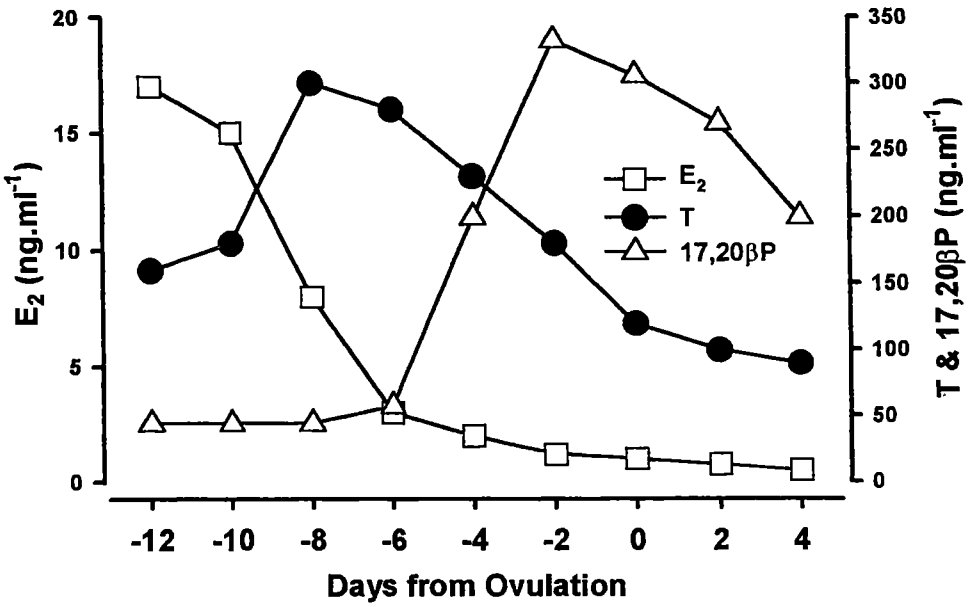


Figure 1.12 Typical plasma levels of 17β-estradiol (E_2), testosterone (T) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) prior to and during the periovulatory period in a female salmonid (Adapted from Fostier et al., 1983 and Van Der Kraak et al., 1984).

1.4.iii Induced maturation

Although captive salmonids typically exhibit normal reproductive development (excluding the behavioural aspects of gamete release), the final stages of reproductive development and associated endocrine changes can also be induced or accelerated by administration of super-active analogues of mammalian or piscine luteinizing hormone-releasing hormone (LHRHa) (reviewed by Donaldson and Devlin, 1996). These compounds stimulate pituitary manufacture and release of GtH-II and are particularly effective when applied in combination with DA antagonists which help to reduce DA's inhibitory tone on GtH-II release (reviewed by Peter et al., 1993).

1.5 The Present Study

In view of the likelihood that Tasmanian water temperature profiles will constrain efforts to phase-shift reproduction in local stocks of Atlantic salmon, and current concerns about the risk of global climate change, it becomes critically important to understand the potential effects of temperature change on the animal's reproductive processes, in order to ensure the maintenance of a profitable and sustainable Atlantic salmon farming industry in Tasmania. Therefore, following from the reports of Pankhurst et al., (1996) and Pankhurst and Thomas (1998), work was conducted to ascertain whether the responses of female Atlantic salmon to elevated temperatures resembled those of rainbow trout, and in order to clarify the endocrine mechanisms involved, experiments were conducted to investigate:

1. The dynamics of oocyte growth and ovarian steroidogenesis during vitellogenesis in Tasmanian Atlantic salmon. There has been only limited characterisation of reproductive development in southern hemisphere stocks of salmonids (eg. Estay et al., 1995, 1998; Pankhurst et al., 1996).

2. The effect of autumn temperature manipulation on ovulation and ovarian steroidogenesis in maturing female Atlantic salmon. A previous study in Atlantic salmon (Taranger and Hansen, 1993) reported inhibition of ovulation at elevated temperatures. However, as no endocrine data were presented, the endocrine correlates of inhibited ovulation had not been characterised in Atlantic salmon.

3. The effect of autumn temperature manipulation on ovulation and ovarian steroidogenesis in female Atlantic salmon challenged with injection of LHRHa. The responsiveness of Atlantic salmon to LHRHa at typical spawning temperatures is understood (eg. Crim et al., 1983; Taranger et al., 1992) but it was not known whether the response to LHRHa of Atlantic salmon at high temperatures was similar to that of rainbow trout where the ovulatory response to LHRHa injection was delayed but not inhibited (Pankhurst and Thomas, 1998).

4. The effects of temperature shifts and ramp-down on ovulation, ovarian steroidogenesis and fertilisation success in un-manipulated female Atlantic salmon

and in fish challenged with injection of LHRHa and/or 17α-hydroxyprogesterone (17P). Taranger et al., (2000) recently reported the value of temperature reduction in promoting ovulation in female Atlantic salmon but in the absence of endocrine data, the mechanisms were unclear. In addition, King and Young (2001) have reported that spermiation of male Atlantic salmon in response to LHRHa treatment can be enhanced by co-administration of 17P the precursor of the maturation-inducing hormone, 17,20βP, but the effects in female Atlantic salmon were unknown.

5. The effect of summer and autumn temperature manipulation on vitellogenesis and egg and larval quality in female Atlantic salmon. Pankhurst et al., (1996) suggested that vitellogenesis in rainbow trout was unaffected by temperature elevation, whereas a later study (Pankhurst and Thomas, 1998) identified some reduction in oocyte size in association with elevated holding temperature. Similarly, the likely impacts of elevated temperature on vitellogenesis in Atlantic salmon were unclear.

Estay et al., (1998) observe that a possible reason that the literature is dominated by work on rainbow trout is the hardiness and ease of management of that species under laboratory conditions. Therefore, other than in the better-resourced Norwegian institutions, studies on adult Atlantic salmon are relatively rare. However, during the present study, interaction with SALTAS provided an opportunity for experimentation with substantial numbers of large (and valuable) Atlantic salmon broodstock under commercial production conditions. As a consequence, the outcomes of the present study have particular relevance for the development and expansion of the Tasmanian Atlantic salmon farming industry.

Chapters 2-6 are presented in the form that they were prepared for publication (see below). This has resulted in some planned repetition of material in introductory and methods sections.

Chapter 2; King, H.R. and Pankhurst, N.W., In prep. Ovarian growth and plasma sex steroid profiles during vitellogenesis in Tasmanian female Atlantic salmon (*Salmo salar*).

Chapter 3; King, H.R. and Pankhurst, N.W. The effect of autumn temperature manipulation on ovulation and ovarian steroidogenesis in maturing female Atlantic salmon (*Salmo salar*) in Tasmania.
Published as:
King, H.R. and Pankhurst, N.W., 2000. Ovulation of Tasmanian Atlantic salmon maintained at elevated temperatures: implications of climate change for sustainable industry development. In: B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson and S.O. Stefansson (Eds), Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish. John Grieg A/S, Bergen, pp. 396-398.

Chapter 4; King, H.R., and Pankhurst, N.W., In prep. Effect of maintenance at elevated temperatures on ovulation and LHRHa responsiveness in Tasmanian female Atlantic salmon (*Salmo salar*).

Chapter 5; King, H.R., and Pankhurst, N.W., In prep. Short-term temperature reduction promotes ovulation and restores LnRHa responsiveness in female Atlantic salmon (*Salmo salar*) maintained at elevated temperatures.

Chapter 6; King, H.R., Pankhurst, N.W. and Watts, M., In prep. Effect of elevated summer temperatures on steroid production, vitellogenesis and egg quality in Tasmanian female Atlantic salmon (*Salmo salar*).

Ethical clearance for the work conducted throughout this study was provided under permit No. A5693 University of Tasmania, Tasmanian Animal Experimentation Ethics Committee.

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CHAPTER 2

OVARIAN GROWTH AND PLASMA SEX STEROID PROFILES DURING VITELLOGENESIS IN TASMANIAN FEMALE ATLANTIC SALMON (*SALMO SALAR*)

2 OVARIAN GROWTH AND PLASMA SEX STEROID PROFILES DURING VITELLOGENESIS IN TASMANIAN FEMALE ATLANTIC SALMON (*SALMO SALAR*)

2.1 Introduction

Salmonids are generally recognised as autumn and winter spawners, although stocks from the higher northern latitudes may reproduce in spring or even summer (reviewed by Scott, 1990). The timing of sexual maturation is, for the most part, influenced by photoperiod (reviewed by Bromage et al., 1993) which is believed to entrain an endogenous rhythm of reproductive development via pineal melatonin secretion (Porter et al., 1999, 2001). As a consequence, populations of salmonids which are transplanted to southern latitudes spawn during the austral autumn and winter, 6 months out-of-phase with their northern hemisphere counterparts (Scott, 1990). For example, the population of Atlantic salmon (*Salmo salar*) of Nova Scotian origin which forms the foundation of the salmon farming industry in Tasmania, Australia, is now recognised as an austral autumn (May) spawning stock (Jungalwalla, 1991).

Although the spawning patterns of southern hemisphere salmonids are generally understood to match those of northern hemisphere stocks (albeit 6 months out-of-phase), more systematic studies of reproductive development in southern hemisphere stocks are lacking. In contrast, ovarian development and associated changes in the patterns of circulating reproductive hormones have been described in detail for a number of northern hemisphere stocks. The majority of studies relate to the rainbow trout (*Oncorhynchus mykiss*) (eg. Campbell et al., 1980; Scott et al., 1980; Bromage et al., 1982; Scott and Sumpter, 1983; Schulz, 1984, Springate et al., 1985; Fostier and Jalabert, 1986; Bromage and Cumaranatunga, 1988; Tyler et al., 1990), but a number of studies have also been conducted on the charrs (*Salvelinus* spp.) (eg. Goetz et al., 1987; Mayer et al., 1992; Foster et al., 1993; Frantzen et al., 1997), Atlantic salmon (eg. Crim et al., 1986; Andersen et al., 1991; Cotter et al., 2000) and a variety of Pacific salmon species (*Oncorhynchus* spp.) (eg. Sower and Schreck, 1982; Dye et al., 1986; Slater et al., 1994). However, similar southern hemisphere studies are essentially restricted to a single description of reproductive development

in a Chilean stock of coho salmon (*O. kisutch*; Estay et al., 1995, 1998) while limited data has been presented on ovarian development and gonadal steroid production in a Tasmanian stock of rainbow trout (Pankhurst et al., 1996) and a Chilean population of Atlantic salmon (Estay et al., 1995). As a consequence, the information required as a basis for studies of reproduction in southern hemisphere salmonids, and in particular Atlantic salmon in Tasmania, is lacking and the details of reproductive development in those stocks can, at best, only be inferred by extrapolation from northern hemisphere data. This is problematic in that transplanted stocks frequently experience environmental conditions which differ markedly from those of their place of origin. For example, Atlantic salmon farmed in Tasmania typically experience seawater temperatures in the range 8-18°C which promote rapid growth and maturation as grilse (i.e. as 2-year-olds after 1 winter of seawater residence), whereas their Nova Scotian relatives are recognised as a late maturing stock (Jungalwalla, 1991). Similarly, the possibility of genetic change in transplanted populations limits the application of northern hemisphere data. In this regard, the Tasmanian population of Atlantic salmon originated from a small number matings conducted in the mid 1960s (ie. approximately 12 generations ago) and, relative to the Canadian ancestral population, some genetic change has been noted (Reilly et al., 1999).

The present study was conducted to provide a contextual basis for further work on the effects of elevated water temperatures on reproduction in Tasmanian Atlantic salmon, and on the manipulation of reproduction in culture. Fish were sampled from sea cages during the vitellogenic phase of reproductive development and plasma levels of gonadal steroids and a range of indices of ovarian development were measured over the austral summer-autumn period.

2.2 Materials and Methods

2.2.i Stock

Samples were collected from female Atlantic salmon maturing for the first time as 2-year olds (ie. after 1 year of seawater residence). The stock was maintained by Aquatas Pty. Ltd. (Margate, Tasmania) at the company's Northwest Bay operations (upper D'Entrecasteaux Channel, Tasmania) and held under commercial culture conditions where they experienced natural photoperiod and temperature regimes. Sampling commenced in October 1997 and ended in late March 1998 (immediately

prior to the return of the fish to freshwater to facilitate final maturation and spawning in late April and early May). At approximately monthly intervals (29-35 days), 10 fish were sampled at random from a sea-cage population using the fishing method developed by Thomas (1998) where, in order to avoid stress to the remainder of the population, fish were deprived of food overnight and a 15-20g 'Halco' lure attached to >15 kg breaking strain fishing line was used to capture individuals. Retrieval of the animals typically took place in 30 to 60 seconds. Immediately following capture, fish were killed by a blow to the head, blood samples were collected (see following section) and the fish were placed on ice for transfer to the laboratory and subsequent assessment of reproductive state.

2.2.ii Blood sampling and measurement of plasma steroids

Blood samples were taken from each fish by puncture of the duct of Cuvier (Lied et al., 1975) using heparinized (lithium heparin) syringes and 22G needles. Syringes were placed on ice for transfer to the laboratory where, after centrifugation, the resulting plasma was stored at -20°C prior to analysis of steroid hormone levels. The levels of E_2 and T in aliquots of thawed plasma were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Carragher (1992). Extraction efficiency (mean recovery of ^{3}H-labelled steroid from triplicates of a plasma pool) was 65 and 86% for E_2 and T respectively and values for each steroid were adjusted accordingly. Interassay variability measured using aliquots of a pooled internal standard was (%CV(n)) 5.6(2) and 14.3(2) for E_2 and T respectively.

2.2.iii Assessment of gonadal development

The total body weight (± 0.05 kg) and total gonad weight (± 0.1 g) were measured and gonadosomatic index calculated (GSI; total ovary weight expressed as a percentage of total body weight). A sample of ovarian tissue was removed from one ovary and placed in a glass petri dish containing an isotonic saline solution and dissected to separate individual follicles. Thereafter, the diameters of 20 oocytes per female were determined using the image analysis technique described by Morehead et al. (1998). Oocytes of less than 500 μm diameter were ignored as they were judged to be previtellogenic and were therefore not expected to be representative of

the vitellogenic cohort of oocytes at the commencement of sampling (Sumpter et al., 1984; Bromage and Cumaranatunga, 1988; Tyler et al., 1990).

2.2.iv Data analysis and statistical comparisons

Using an approach similar to that of Tyler et al. (1990), variability in oocyte diameter over the course of the study was examined by construction of oocyte size-frequency distributions where, for the median fish on each sample date, the numbers of oocytes within 100 μm size classes were plotted as percentages of the total number of oocytes measured. Similarly, the variability in oocyte size within the ovaries of each female at each sample date was assessed by expressing the size of the oocytes for each fish as a percentage of the median oocyte size class for that fish. The data for all 10 fish were then combined for each sample interval. Here the sizes of oocytes were expressed in terms of their volumes as volume provides a representation of mass and thereby, a more physiologically relevant and direct indication of the yolk reserves contained in an oocyte (Tyler et al., 1990).

Changes in mean body weight, GSI, oocyte diameter and plasma levels of E_2 and T were assessed by one-way ANOVA and Tukey's HSD tests while relationships between E_2 levels and oocyte sizes were examined by linear and non-linear regression techniques. All analyses were conducted using the SYSTAT 8.0 for Windows computer package.

2.3 Results

The mean body weight of maturing female Atlantic salmon increased significantly ($F_{(5,54)}$=8.2, P<0.001) from approximately 3.4 kg at the commencement of sampling to 5.4 kg prior to transfer to freshwater (Figure 2.1). Concomitantly, mean GSI increased significantly ($F_{(5,54)}$=375.8, P<0.001) from approximately 0.5% in October to 17% in late March (Figure 2.1). Increases in GSI occurred between the November and December samples and most markedly following the January sample, whereas there was no significant change in GSI between the October and November samples, and the December and January samples.

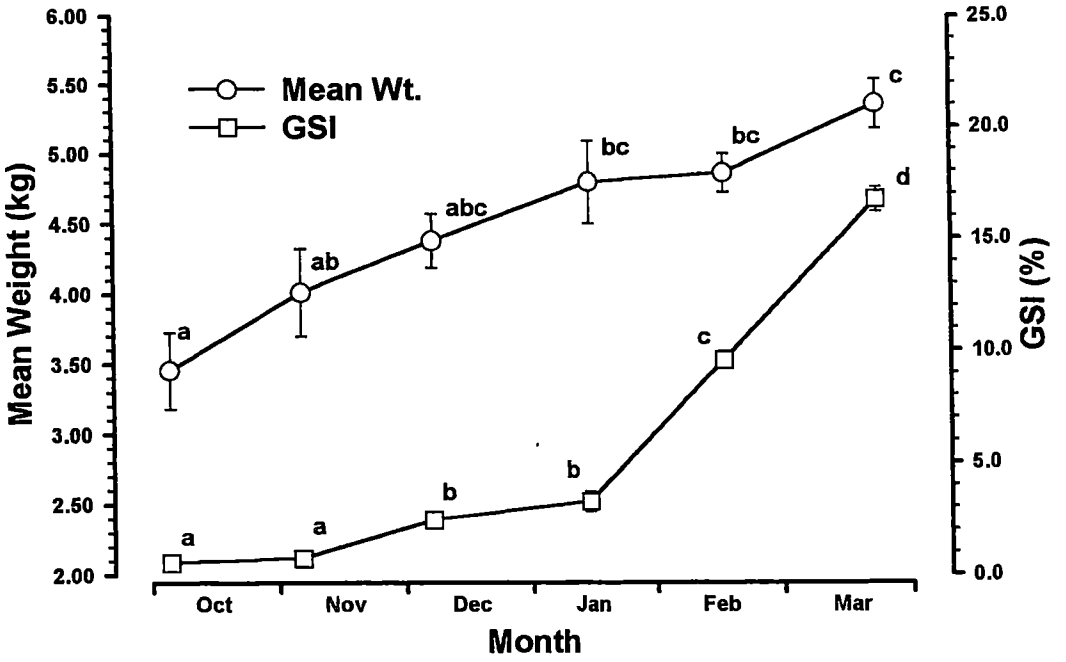

Figure 2.1 Change in mean (± SEM) weight (O) and gonadosomatic index (GSI, □) of Tasmanian female Atlantic salmon during vitellogenesis. Points with the same superscript are not significantly different ($P > 0.05$).

Mean oocyte diameter increased significantly between October and March ($F_{(5,54)}$=224.6, $P<0.001$), increasing in a linear fashion from approximately 1.3 mm to 5.4 mm (Figure 2.2). The greatest increases in oocyte diameter occurred between the November and December samples when mean diameter increased by 1.0 mm from 1.6 to 2.6 mm, and between the January and February samples when mean diameter increased by 1.8 mm from 3.1 to 4.8 mm. In contrast, the rate of oocyte growth during the December-January sampling interval was reduced relative to that of the preceding and subsequent intervals.

In accordance with changes in oocyte diameter, mean oocyte volume also changed significantly ($F_{(5,54)}$=207.1, $P<0.001$) over the sampling period, increasing from approximately 1.2 mm^3 at the commencement of sampling to approximately 84 m^3 at the conclusion of sampling (Figure 2.3). The greatest increase in oocyte volume occurred between the January and March samples when oocyte volume increased more than 5-fold.

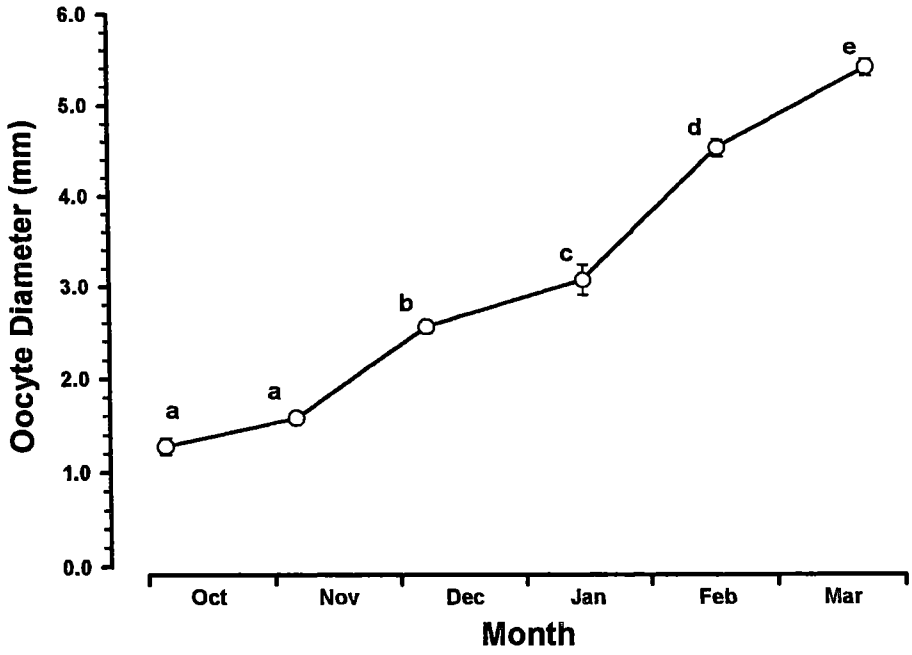

Figure 2.2 Change in mean (± SEM) diameter of oocytes from Tasmanian female Atlantic salmon during vitellogenesis. Points with the same superscript are not significantly different ($P > 0.05$).

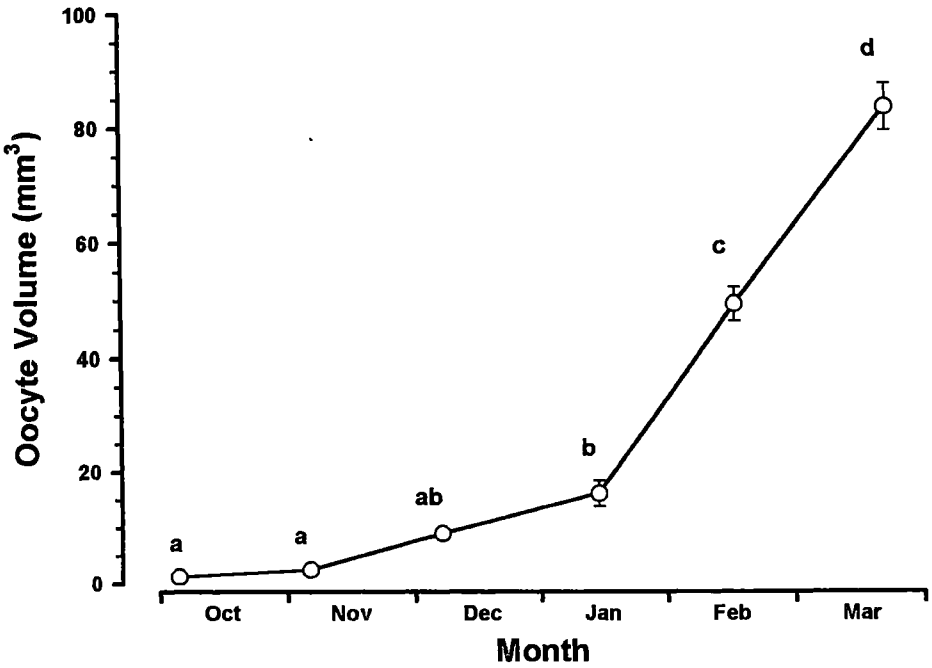

Figure 2.3 Change in mean (± SEM) volume of oocytes from Tasmanian female Atlantic salmon during vitellogenesis. Points with the same superscript are not significantly different ($P > 0.05$).

Variation in the diameter of oocytes was greatest in the January and February samples and lowest in the October, November and March samples (Figure 2.4), whereas relative oocyte volume was most variable in the October to January samples where there was up to a 6-fold difference in oocyte volume within the ovaries of any one fish (Figure 2.5). At later sample times, however, the variation was reduced such that oocytes sampled in March displayed a less than 2-fold variation in volume.

Plasma levels of E_2 changed significantly over the course of the study ($F_{(5,54)}$=55.0, P<0.001), increasing from basal levels at the commencement of sampling of ~ 3 $ng.ml^{-1}$, to greater than 21 $ng.ml^{-1}$ at the completion of sampling (Figure 2.6). E_2 levels did not change until March when there was a marked increase. Plasma T also increased significantly ($F_{(5,54)}$=91.3, P<0.001) from approximately 3 $ng.ml^{-1}$ at the commencement of sampling, to almost 20 $ng.ml^{-1}$ at the final sampling (Figure 2.6). As for E_2, the greatest increase in plasma T occurred between February and March when a 3-fold increase in hormone level occurred. February values were also significantly higher than those found in October and November.

Individual E_2 levels displayed a weak (R^2=0.549) but significant ($F_{(1,57)}$ = 69.5, P<0.001) linear correlation with individual oocyte diameters (Figure 2.7a). However, non-linear regression revealed a stronger (R^2=0.912) exponential relationship between E_2 levels and oocyte diameters described by the equation:

$$\mathbf{y = 3.154 + 0.003e^{1.562x}}$$

where x and y represent oocyte diameter and E_2 level respectively.

E_2 levels displayed a stronger (R^2=0.717), significant ($F_{(1,57)}$ = 144.3, P<0.001) linear correlation with individual oocyte volumes (Figure 2.7b). Nonetheless, here again, non-linear regression yielded a stronger (R^2=0.913) exponential relationship described by the equation:

$$\mathbf{y = 1.904 + 1.095e^{0.032x}}$$

where x and y represent oocyte volume and E_2 level respectively. Similarly, E_2 levels also displayed a significant ($F_{(1,57)}$ = 143.9, P<0.001) linear correlation (R^2=0.717) with GSI (Figure 2.7c) while non-linear regression yielded a stronger (R^2=0.888) exponential relationship ($y = 0.419 + 2.12e^{0.132x}$).

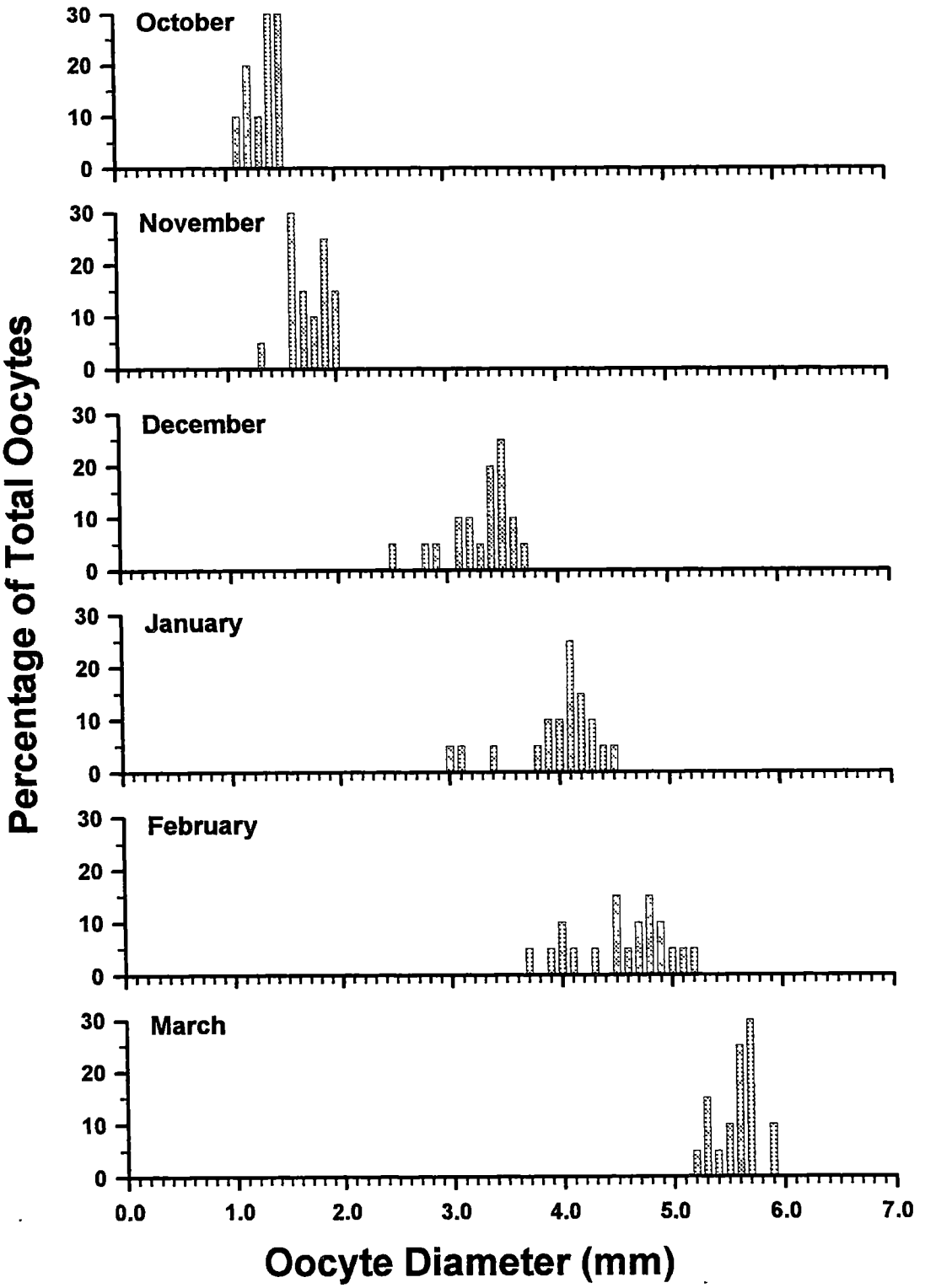

Figure 2.4 Change in the diameter of oocytes (measuring 0.5mm or greater) in the ovaries of Tasmanian female Atlantic salmon during vitellogenesis. The proportion of oocytes in each 100μm diameter size class is expressed as a percentage and plots represent the median for each sampling.

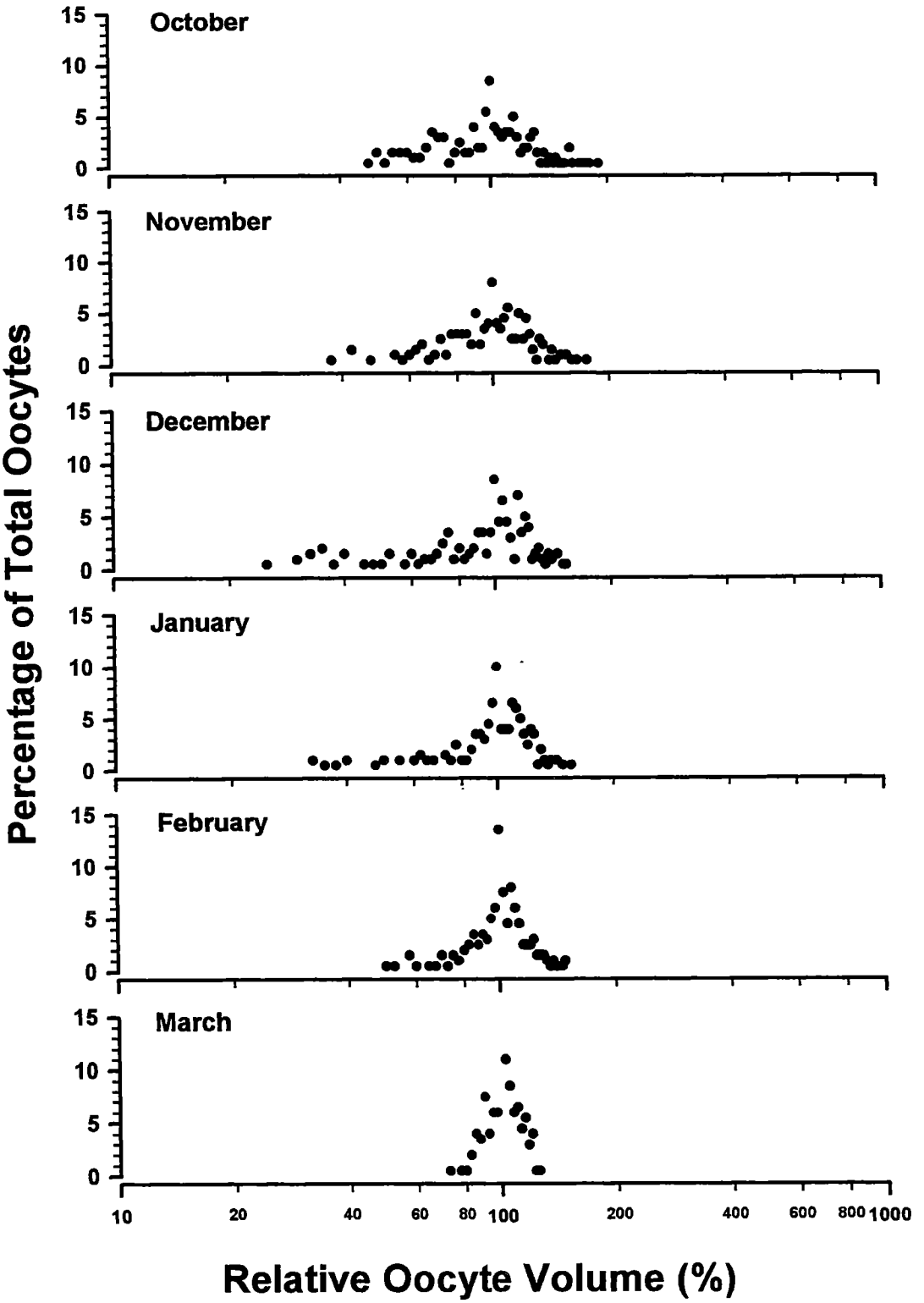

Figure 2.5 Variation in oocyte volume in the ovaries of Tasmanian female Atlantic salmon during vitellogenesis. Volumes were plotted as a percentage of the mean oocyte volume and each monthly plot represents a composite of all 10 females centred around the median oocyte size class.

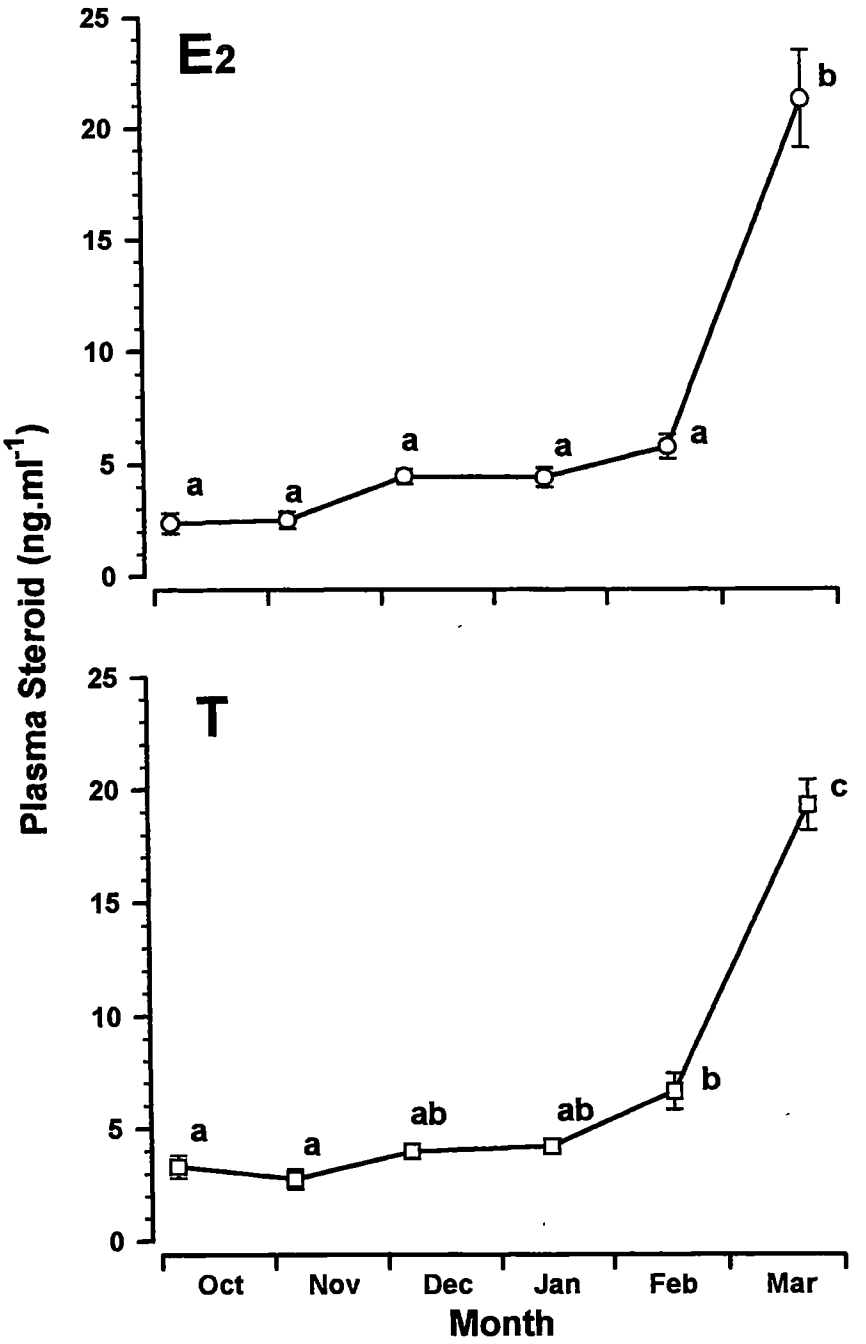

Figure 2.6 Change in mean (± SEM) plasma E_2 (O) and T (□) levels in Tasmanian female Atlantic salmon during vitellogenesis. Points with the same superscript are not significantly different ($P > 0.05$).

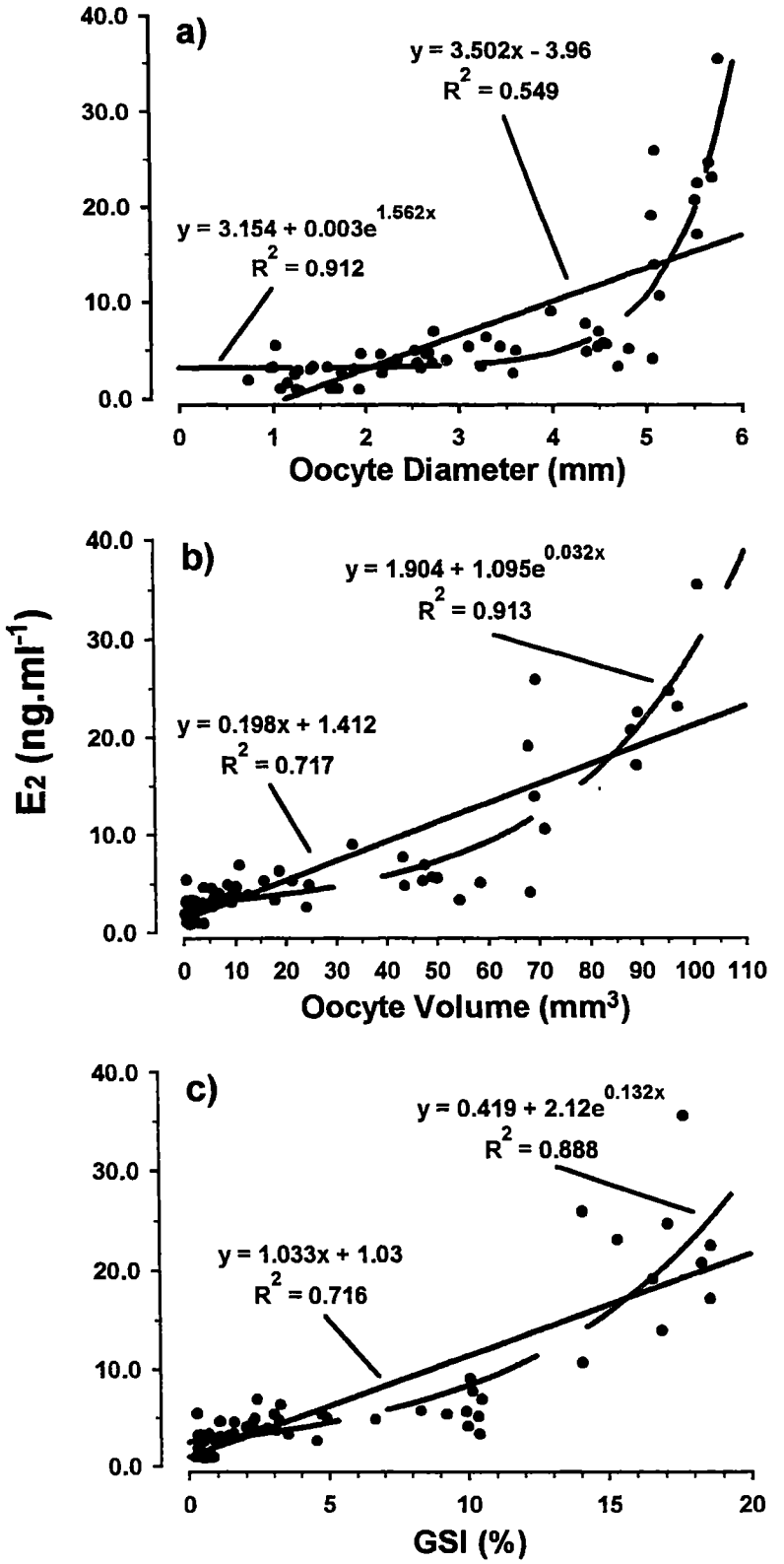

Figure 2.7 Scatter plots of individual oocyte diameter (a), oocyte volume (b) and GSI (c) values versus individual plasma E_2 levels for Tasmanian female Atlantic salmon during vitellogenesis. Best fit linear and exponential regression equations are represented by solid and broken lines respectively.

2.4 Discussion

During the present study, the weight of Tasmanian female Atlantic salmon increased from approximately 3.4 kg to 5.4 kg, consistent with the typical October to March range in harvest weights for Tasmanian Atlantic salmon reported by Jungalwalla (1991). At the same time, GSI underwent an approximately 35-fold increase reaching a level of 17% at the completion of sampling. Tyler et al. (1990) reported an increase in GSI from 0.4% to 20% in a 9-month study of rainbow trout which concluded at ovulation. Bearing in mind the relatively later start and earlier conclusion of the present study (ie. approximately 4-6 weeks prior to ovulation), the rate of gonadal growth observed is generally consistent with that reported by Tyler et al. (1990) and in other studies where maximum pre-ovulatory GSIs in salmonids have been reported to range from 20 to 25% (reviewed by Tyler, 1991). In a more direct comparison with other southern hemisphere salmonid stocks, Pankhurst et al. (1996) reported a maximum pre-ovulatory GSI of 15.8% in Tasmanian stock of rainbow trout. Similarly, Estay et al. (1998), in a study of Chilean coho salmon which generally parallelled the present study, reported a maximum GSI of approximately 17% at ovulation. At all points where the bimonthly sampling of Estay et al. (1998) corresponded to that of the present study, the Tasmanian salmon GSI exceeded that of the Chilean fish. For example, the January GSI of the Chilean coho salmon was 1.36% (± 0.29) whereas, in the present study this figure was exceeded one month earlier. In this context, it is noteworthy that Estay et al. (1995) also reported (without presenting any data) a trend towards relatively greater GSI in a Chilean stock of Atlantic salmon compared to simultaneously sampled coho salmon However, as the Atlantic salmon were one year older than the coho salmon (ie. maturing for the first time as 3-year-olds after an additional year of seawater residence) the significance of this trend is difficult to interpret and may simply reflect earlier reproductive development on the part of the older fish. Bromage and Cumaranatunga (1988) have observed that 3-year-old rainbow trout commenced exogenous vitellogenesis 8-10 months prior to spawning whereas the same process occurred over only 4-6 months in 2-year-old fish.

Concomitant with increases in GSI, mean oocyte diameter also underwent a 4-fold increase (equivalent to a 60 to 70-fold increase in volume). Here again, the observations are in general agreement with those reported elsewhere (eg. Springate et

al., 1985; Bromage and Cumaranatunga, 1988; Tyler et al., 1990; Estay et al., 1995, 1998; Pankhurst et al., 1996). The relatively greater body size of the present females might possibly account for any trend towards greater oocyte size in the present study. Springate et al. (1985) and Tyler et al. (1990) reported final body weights of approximately 1.5 kg in their respective studies of female rainbow trout, while Estay et al. (1998) reported a final weight of 3.52 kg in their female coho salmon. In contrast, the female Atlantic salmon observed during the present study reached 5.4 kg at the end of sampling. As a result, a greater oocyte diameter might be expected consistent with the observations of a number of authors (reviewed by Thorpe et al., 1984) and in particular, those of Springate et al. (1985) where female size was positively correlated with oocyte diameter, and provided a means for maintenance of relative fecundity in smaller fish. Similarly, Thorpe et al. (1984) observed a significant positive correlation between parental fork length and egg diameter. Alternatively, the relatively greater oocyte size of Atlantic salmon in the present study may reflect more intrinsic stock differences, as other authors have presented data which tends to refute any relationship between female size and egg size. In a study of four farmed stocks of Atlantic salmon, Thrush and Bromage (1991) found egg diameter to be only poorly related to female weight. Half of the groups investigated showed no correlation, while the combination of all data from all stocks yielded an R^2 value of only 0.182. Similarly, Kaeriyama et al. (1995) suggested that egg size was stable within a cohort or population of sockeye salmon (*Oncorhynchus nerka*) irrespective of variations in body size.

Tyler et al. (1990) showed that the growth rates of rainbow trout oocytes during vitellogenesis were highly variable. Oocyte diameters for the median female sampled exhibited a range of up to 2,500 μm approaching the mid point of sampling, reducing to only 500 μm prior to ovulation. Prior to presenting their own apparently contrasting findings, Tyler et al. (1990) suggested that the literature, in particular the report of Springate et al. (1985), tended to imply that oocyte growth during vitellogenesis was uniform so that, at any point prior to and including ovulation, all vitellogenic oocytes would be of a similar size. However, this interpretation of the work of Springate et al. (1985) is confusing as those authors reported on only a single set of vitellogenic samples collected at approximately 14 days prior to ovulation; a time when, according to the work of Tyler et al. (1990), oocyte size

could be expected to be relatively uniform. Furthermore, Springate et al. (1985) did not present any data and made no comments on the sizes of oocytes prior to ovulation, but rather, reported that on the basis of histological characteristics, the majority of developing oocytes in their limited pre-ovulatory sampling were at similar developmental stages. During the present study, oocyte diameter displayed a range of up to 1,600 μm around the mid point of sampling, reducing to 800 μm at the completion of sampling. The present assessment of variations in oocyte volume is consistent with that reported by Tyler et al. (1990). Approximately half-way through vitellogenesis, those authors observed a 5-fold variation in oocyte volume while at a corresponding point in the present study, variation in oocyte volume was approximately 6-fold. Similarly, a reduction in variability of oocyte volume to less than 2-fold occurred with oocyte growth in both the present study and that of Tyler et al. (1990). From this it would appear that there is no inconsistency in the literature with regard to the variability of oocyte size during vitellogenesis, and the results of the present study are consistent with those of both Tyler et al. (1990) and Springate et al. (1985) in that oocyte size was variable during the earlier stages of vitellogenesis and became more consistent as ovulation approached.

Plasma levels of the gonadal steroid E_2 and its precursor T were also broadly consistent with those reported elsewhere. Both hormones were at levels below 5 $ng.ml^{-1}$ in October but reached approximately 20 $ng.ml^{-1}$ by March. This matches the magnitude of increase reported in a variety of salmonid and non-salmonid species where plasma levels of E_2 have been recorded in relation to ovarian growth and development (eg. Scott et al., 1980; Fostier et al., 1983; Scott and Sumpter 1983; Schultz, 1984; Fostier and Jalabert, 1986; Younson et al., 1988; Oppen-Berntsen et al., 1994, Taranger et al., 1999) in accordance with the recognised role of E_2 in stimulating hepatic synthesis of the yolk protein precursor vitellogenin (reviewed by Mommsen and Walsh, 1988; Tyler, 1991; Specker and Sullivan, 1994). However, it should be recognised that some notable exceptions to the general picture of salmonid E_2 production exist. For example, Bromage et al. (1982) observed peak E_2 levels of only approximately 5 $ng.ml^{-1}$ during a study of photoperiod effects on the control of ovarian development in rainbow trout while Mayer et al. (1992) and Frantzen et al. (1997) reported peak E_2 levels of only 3.5 $ng.ml^{-1}$ and 11 $ng.ml^{-1}$ respectively in their studies of reproductive development in Arctic charr. It remains unclear whether these

exceptions result from stock, species and/or year-to-year variability in salmonid E_2 production, although it should also be noted that, irrespective of the absolute levels of E_2 measured, the relative magnitude of the seasonal changes in E_2 observed during the above studies conformed to the general pattern reported elsewhere. With respect to other southern hemisphere salmonids, the steroid levels found in Atlantic salmon in the present study are also consistent with those reported in Tasmanian rainbow trout (Pankhurst et al., 1996) and Chilean coho salmon (Estay et al., 1995, 1998) but tend to differ from those reported in a Chilean Atlantic salmon stock, where a maximal plasma level of E_2 of only ~5 $ng.ml^{-1}$ was observed in January, approximately 4 months prior to ovulation (Estay et al., 1995). Here again, the fact that the Chilean Atlantic salmon were a year older may account for the differing steroid production in that the longer vitellogenic phase of the older fish (Bromage and Cumaranatunga, 1988) could permit the necessary final oocyte size to be achieved at a lower rate of growth which would in turn, require lower levels of E_2-stimulated vitellogenin synthesis. However, Oppen-Berntsen et al. (1994) also studied an older population of Norwegian Atlantic salmon but observed levels of E_2 production similar to those of the present study. Therefore, the Chilean results may simply reflect the peculiarities of the Chilean stock or growing conditions.

In addition to absolute level, and magnitude of change in level, an important characteristic of hormone production is rate of change in hormone level. In this context, Scott et al. (1980) presented data depicting a linear increase in circulating E_2 levels in rainbow trout while Schulz (1984) observed a strong linear relationship ($R=0.92$) between oocyte diameter and serum E_2 level in the same species, implying a linear increase in serum E_2 level over time. Other authors have reported linear correlations between GSI and plasma E_2 levels in female salmonids. For example, Kagawa et al. (1983) observed a linear increase in the GSI of Amago salmon and reported (without providing details of any statistical analysis) that E_2 level and GSI correlated well during vitellogenesis, thereby implying a linear increase in circulating E_2. Following from this, Oppen-Berntsen et al. (1994) reported an "estimated reproduced correlation" (derived from principal components analysis) of 0.996 between plasma levels of E_2 and GSI in Norwegian Atlantic salmon. These authors did not present any GSI data. However, their E_2 data were generally indicative of a linear increase. During the present study, E_2 levels were essentially unchanging until

the final sample interval when a 4-fold increase occurred such that the linear correlation between oocyte diameter and E_2 level was relatively weak (R^2=0.55) whereas, a stronger non-linear correlation (R^2=0.91) confirmed an exponential increase in E_2 levels. Furthermore, neither GSI or oocyte volume increased in a linear fashion. Both parameters changed relatively little over the first three sampling intervals then underwent successive 2 to 3-fold increases during the last two sampling intervals. As a result, both GSI and oocyte volume exhibited a closer linear correlation (R^2=0.72) with E_2 levels than did oocyte diameter although improvements in the level of correlation (R^2=0.89 and 0.91 for GSI and oocyte volume respectively) were provided by the non-linear relationship which better reflected the exponential increase in E_2 levels. In this context, a number of other authors have reported sharp late-vitellogenic increases in circulating E_2 in species such as rainbow trout (eg. Bromage et al., 1982; Scott and Sumpter, 1983) and Arctic charr (Meyer et al., 1992; Frantzen et al., 1997). However, only Cotter et al. (2000) have reported patterns of increase in the levels of E_2 (and T) in Atlantic salmon similar to those observed in the present study. In a population of Irish Atlantic salmon examined by these authors, plasma levels of both E_2 and T were consistently low (< 3 $ng.ml^{-1}$) during the 10-month period spanning winter, spring and summer (November – August) but exhibited an approximately 5-fold increase over the 1-month period (August – September) which corresponds to seasonal phase of the final sample time of the present study.

In view of the variability between the studies conducted hitherto, it is difficult to assess the significance of the non-linear increases in parameters such as GSI and plasma E_2 observed in the present study. Taking into account the report by Cotter et al. (2000), it could be concluded that the present results represent the norm for Atlantic salmon. Alternatively, considering the results of studies such as those of Oppen-Berntsen et al. (1994) and Kagawa et al. (1983), where parameters such as E_2 level and GSI were reported to be closely correlated and/or increase in a linear fashion, it could be concluded that the relatively late increases in those same parameters in the present study are possibly indicative of earlier impairment of reproductive development. In this context, Springate et al. (1985) demonstrated that reduced ration resulted in reductions in oocyte diameter and serum levels of T and vitellogenin (indicated by serum calcium levels). However, during the present study,

it is highly unlikely that feed input on a commercial operation could have been reduced to an extent sufficient to elicit a consistent effect at the population level In addition, it is noteworthy that Springate et al. (1985) only observed reduced serum T and calcium towards the end of vitellogenesis in feed-restricted fish and, while they presented no statistical analysis, those variables appeared not to differ between full-ration and half-ration groups during the preceding 5 months. Therefore, if the present study is genuinely indicative of retardation of reproductive investment it is more likely that the results reflect some kind of stress effect. In this regard, the effect of stress on plasma levels of gonadal steroids is recognised in many fish species where it results in depression of androgen and/or estrogen levels (reviewed by Pankhurst and Van Der Kraak, 1997). If retarded oocyte development and endocrine activity reflect stress, there are several candidates under Tasmanian conditions:

1. Elevated water temperature. Generally, maximum seawater temperatures are approached over the late austral summer and temperature is known to affect reproductive development in all species including salmonids (reviewed by Van Der Kraak and Pankhurst, 1997). However, while elevated temperatures can inhibit maturational processes (Gillet, 1991; Taranger and Hansen, 1993; Pankurst et al., 1996; Pankhurst and Thomas, 1998), the effects of elevated temperature on vitellogenesis are less well understood. Elevated winter temperatures (10°C) increased the hepatic vitellogenic response relative to that observed at lower temperatures (3°C) in Atlantic salmon post-smolts *in vivo* (Korsgaard et al., 1986) and *in vitro* yolk sequestration by developing rainbow trout oocytes has been observed to be enhanced at elevated temperatures (Tyler et al., 1987). However, in contrast, Chmilevsky (2000) recently reported arrested gonadal development in rainbow trout exposed to extreme temperatures (22-23°C) while Tveiten et al. (2000) observed a 4-5 week delay in the attainment of peak E_2 and T levels in female wolfish exposed to elevated temperatures (8 and 12°C) relative to those maintained at ambient temperatures (3-4°C). Thus, in any one species, it is unclear at which point the stimulatory effect of elevated temperature on vitellogenesis turns to an inhibitory one. In this regard, Pankhurst et al. (1996) reported essentially unimpaired vitellogenesis in Tasmanian rainbow trout reared at temperatures as high as 21°C but it is not known whether the responses of

Tasmanian female Atlantic salmon to elevated temperatures resemble those of rainbow trout.

2. Amoebic Gill Disease (AGD, *Paramoeba pemaquadensis*). December – January represents the period of peak AGD infestation in Tasmanian post-smolts (Munday et al., 1990) and although 2+ fish generally exhibit acquisition of immunity, re-infection can occur where the level of challenge is sufficiently high (Findlay et al., 1995; Findlay and Munday, 1998). In addition there may be undetected sub-acute effects in 2+ fish.

3. Jelly-fish (eg. *Aurelia* sp., *Cyanea* sp.) blooms. In recent years, interactions between jelly-fish swarms and cage-reared fish have occurred throughout south-east Tasmania (Tasmanian Aquaculture and Fisheries Institute, unpublished) and, mirroring observations elsewhere (eg. Bruno and Ellis, 1985), a variety of acute and sub-acute effects ranging from ~100% mortality to impaired growth have been reported. Significantly, there is anecdotal evidence that some female fish exhibiting signs of jelly-fish damage have also shown signs of follicular atresia (Munday, personal communication) although it is not clear how the levels of atresia compare between jelly-fish affected and unaffected animals.

Assessment of the significance of any possible decline in reproductive investment is further complicated by the fact the later stages of vitellogenesis appeared to progress as normal and, in particular, plasma E_2 and T returned to expected levels at the completion of sampling.

In summary, somatic growth and gonadal development in Tasmanian female Atlantic salmon is broadly consistent both with previous reports of the stock's growth and development as an austral autumn spawning stock, and with the general pattern of salmonid reproductive development. An apparent delay in the expected increases in GSI, oocyte volume and E_2 and T levels may have been indicative of reduced follicular steroidogenic capacity and impaired reproductive investment during the earlier stages of vitellogenesis. However, the evidence was equivocal and as the remainder of vitellogenesis appeared to progress normally, its significance is difficult to asses. This highlights the importance of studying individual stocks and emphasises the potential risks associated with extrapolation from one stock to another, especially

where species are grown outside of their natural range and/or under sub-optimal environmental conditions.

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CHAPTER 3

THE EFFECT OF AUTUMN TEMPERATURE MANIPULATION ON OVULATION AND OVARIAN STEROIDOGENESIS IN MATURING FEMALE ATLANTIC SALMON (*SALMO SALAR*) IN TASMANIA

3 THE EFFECT OF AUTUMN TEMPERATURE MANIPULATION ON OVULATION AND OVARIAN STEROIDOGENESIS IN MATURING FEMALE ATLANTIC SALMON (*SALMO SALAR*) IN TASMANIA

3.1 Introduction

The culture of Atlantic salmon (*Salmo salar*) in Tasmania, Australia is conducted at latitudes (approximately 42°S) where natural water temperatures frequently approach the upper limits of thermal tolerance. Although seawater temperatures generally range between 8 and 18°C (Jungalwalla, 1991), summer temperatures can often exceed 19-20°C for a number of weeks, and peaks above 22°C regularly occur in some areas. This is below the reported lethal temperatures for juvenile Atlantic salmon (approximately 28°C; reviewed by Gibson, 1993) but does coincide with the upper threshold lethal temperature range of 22.0-23.5°C presented by Barton (1996). Nevertheless, the relatively high water temperatures experienced by Tasmanian stocks of farmed Atlantic salmon have, for the most part, tended to favour rapid growth and have made a positive contribution to production efficiency. However, there is growing concern in relation to the likelihood that global temperature change may result in even higher summer temperatures. In Tasmania, this interest has been heightened by recent El Niño- Southern Oscillation (ENSO) events. ENSO occurs in years when the trade winds subside, encouraging the accumulation of warm, moisture-laden air in the eastern Pacific and the development of conditions of high atmospheric pressure in the western Pacific (Allen et al., 1996). High rainfalls result in the east whereas conditions in the west are characterised by drought and, in south-eastern Australia, by elevated temperatures.

Abnormal elevation of temperature is known to affect almost all aspects of reproduction in fish, from gametogenesis to larval development, with initiation of sexual maturation and gonadal growth, and the periovulatory periods being particularly sensitive phases (reviewed by Van Der Kraak and Pankhurst, 1997). In salmonids, elevated temperatures have been reported to reduce post-ovulatory egg viability and sperm volume, and inhibit ovulation. While the effects of elevated temperature are understood to be mediated by the endocrine system, information on

the nature of the physiological mechanisms involved is limited (reviewed by Pankhurst et al., 1996). For example, Gillet (1991) and Taranger and Hansen (1993) reported inhibition of ovulation, in response to exposure to elevated temperature in Arctic charr (*Salvelinus alpinus*) and Atlantic salmon respectively. However, in the absence of endocrine data, the mechanisms involved were unclear. More recently, Pankhurst et al. (1996) and Pankhurst and Thomas (1998) also reported on temperature-related inhibition of ovulation in rainbow trout (*Oncorhynchus mykiss*) and presented endocrine data which suggested an effect on final oocyte maturation (FOM). Plasma testosterone (T) and 17β-estradiol (E_2) levels were unaffected by maintenance of vitellogenic fish at elevated temperatures (Pankhurst et al., 1996) but production of the maturation-inducing steroid 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) in response to injection of luteinizing hormone releasing hormone analogue (LHRHa) was delayed at 18°C relative to 12°C (Pankhurst and Thomas, 1998). In view of the fact that ovarian follicles showed *in vitro* responsiveness to gonadotropin (GtH) in terms of E_2 production , and that LHRHa stimulated increases in plasma T *in vivo* (Pankhurst and Thomas, 1998) it was suggested that the main effect of maintenance at elevated temperature was to delay expression of 20β-hydroxysteroid dehydrogenase (20β-HSD) by granulosa cells.

The present study examined the effect in Atlantic salmon, of transfer from natural temperatures (11-15°C) to temperatures between 6 and 16°C approximately one month prior to the normal time of spawning. The objectives were to assess whether Tasmanian stocks of farmed Atlantic salmon exhibited similar temperature sensitivity in terms of ovulation and egg viability, as other salmonids, and to examine whether the endocrine effects of maintenance at elevated temperatures were consistent with those described previously in rainbow trout. Accordingly, fish underwent regular ovulation checks and blood sampling; plasma was analysed for levels of the gonadal steroids T, E_2 and 17,20βP, and ova from successful ovulations were assessed for fertility and survival.

3.2 Materials & Methods

3.2.i Stock, Husbandry, Temperature and Photoperiod Regimes

SALTAS broodstock (sexually maturing 2+ Atlantic salmon) were transported from Aquatas Pty. Ltd., (Margate, Tasmania) to SALTAS Freshwater Operations (Wayatinah, Tasmania) in late March 1997 and were initially maintained in outdoor concrete raceways supplied with river water at natural temperature (11-15°C). After 10 days, 30 female fish (mean weight 5.9kg) were transferred from natural conditions to temperature-controlled $4m^3$ Rathbun tanks (10 fish per tank) set at final temperatures of 6, 11 and 16°C. Tanks were supplied with partially recirculated water. Biological filtration and water exchanges of 50% per day were employed to prevent the accumulation of toxic metabolites. Oxygen saturation was maintained at 100-120% by the addition of gaseous oxygen via ceramic diffusers. Transfer from natural temperature to 6°C was graded over 5 days. After 60 days the temperature in the 16°C system was reduced by $1°C.day^{-1}$ until a final water temperature of 8°C was achieved. Fish were exposed to a simulated natural photoperiod (42° south) for the duration of the study.

3.2.ii Treatments

24h after transfer to the temperature-controlled systems all fish were anaesthetised (25p.p.m. benzocaine), weighed (± 0.1kg) and tagged by placing visible implant tags (VI Tags, Northwest Marine Technology Inc, Shaw Island, WA) in the left adipose eyelid according to the methods described by Bergman et al. (1992) and Kincaid and Calkins (1992). Blood samples were also taken from each fish by puncture of the duct of Cuvier (Lied et al., 1975) using heparinized (lithium heparin) syringes and 22G needles. After centrifugation, the resulting plasma was stored at -20°C prior to analysis of steroid levels. Anaesthesia and blood sampling was repeated at weekly intervals. Following commencement of the recognised spawning season (early May) fish were also checked for ovulation at 2-3 day intervals. Blood sampling and ovulation checks were continued until all surviving fish had ovulated.

3.2.iii Ova Fertilisation and Incubation

Fish that expressed ova in response to the gentle application of pressure to the abdomen were transferred to a holding tank maintained at 8°C. After 24h at 8°C fish were killed by a blow to the head, towelled dry and ova were expressed into a stainless steel sieve. In order to facilitate ova collection, a 2cm cut was made at the genital papilla. Ova were transferred to a stainless steel bowl and fertilised using pooled milt from 3-4 naturally spermiating males. Ova and milt were gently mixed and water (500ml) was added to ensure sperm activation. After 2 mins, ova were rinsed with clean water then left to water-harden for 60 mins. After water-hardening sample batches (1000-1500) of ova from each female were removed and incubated at 8°C in mesh baskets (4.0cm x 10.0cm x 12.0cm) placed in Heath vertical incubator trays (Marisource Inc., Tacoma, WA). Sub-samples of ova from each female were collected after water-hardening and fertilisation (%) was determined on the basis of first cell division after 120 degree-hours, visualised by treatment with a clearing solution (1:1:1 v/v methanol:acetic acid:water) for 2 mins. Eyed ova survival (%) was observed directly after 250 degree-days.

3.2.iv Steroid Hormone Measurement

Plasma levels of E_2, T and 17,20βP were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Carragher (1992). Extraction efficiency (mean recovery of ^{3}H-labelled steroid from triplicates of a plasma pool) was 63-81, 75-87 and 91-100% for E_2, T and 17,20βP respectively and values for each steroid were adjusted accordingly. Interassay variability measured using aliquots of a pooled internal standard was (%CV(n)) 16.0(8), 14.5(8) and 13.9(8) for E_2, T and 17,20βP respectively.

3.2.v Statistical Analysis

Data were analysed by repeated measures ANOVA, one-way ANOVA and Tukey's HSD tests using the SYSTAT 8.0 for Windows computer package. Proportion data were normalised by arcsin transformation and, where required, steroid data were log-transformed in order to satisfy requirements for homogeneity of variances.

3.3 Results

3.3.i Ovulation and Ova Survival

In fish maintained at 6°C, ovulations commenced 34 days after transfer to temperature-controlled conditions, and all fish ovulated within 27 days (Figure 3.1). Ovulation commenced 3 days later in fish maintained at 11°C and was completed within 31 days. No ovulation occurred in fish held at 16°C prior to temperature

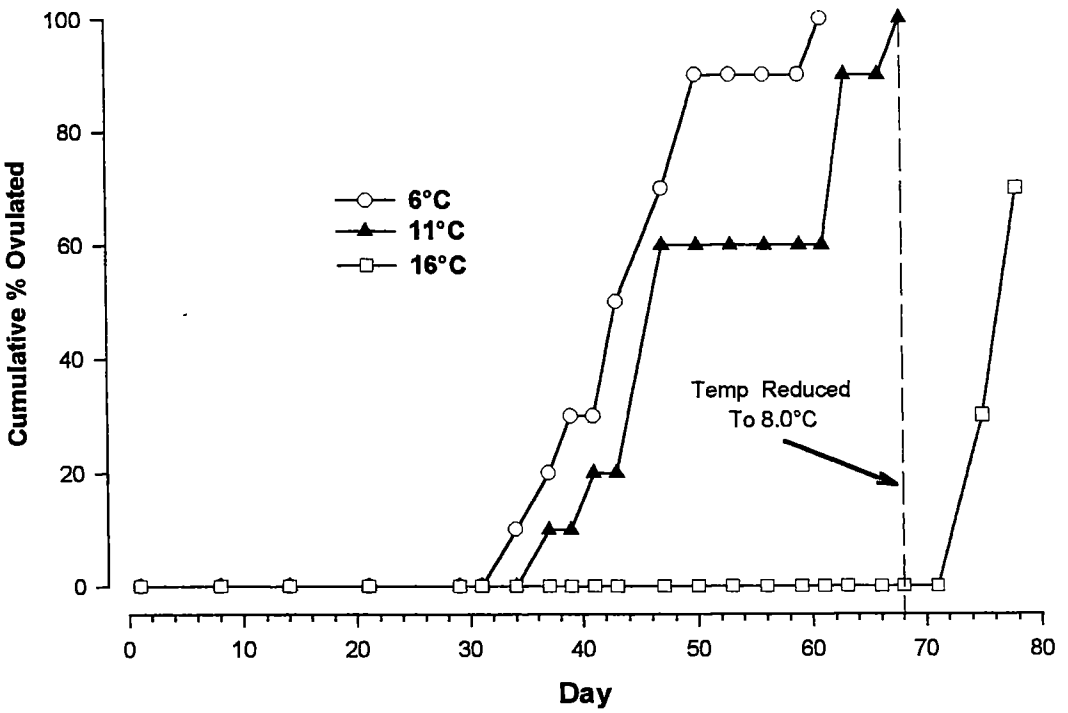

Figure 3.1 Cumulative percentage of fish ovulating at 6, 11 and 16°C (n=10). The water temperature of the 16°C treatment was reduced to 8°C (arrow) on day 68.

reduction. The temperature of 16°C fish was reduced to 8°C by day 68 of the study, and ovulations in fish commenced 7 days later. All surviving fish (7 of the original 10) ovulated within a further 3 days. Ova from fish maintained at 6 and 11°C displayed high fertility while that of ova from fish held at 16°C was significantly lower ($P<0.01$; Table 3.1). Mean eyed ova recovery was highest in ova from fish maintained at 11°C. Eyed ova recovery was lower, but not significantly so ($P>0.05$), at 6°C while the

recovery of eyed ova from fish held at 16°C was significantly lower than that at 11°C but not different from that at 6°C (Table 3.1).

Ova were not maintained beyond 250 degree-days.

Temp.	Fertility (%)	Eyed (%)
6°C	97.3 ± 0.6^{a}	48.4 ± 14.4^{ab}
11°C	97.3 ± 0.9^{a}	82.0 ± 3.7^{a}
16°C	82.5 ± 4.4^{b}	24.7 ± 17.4^{b}

Table 3.1 Percent fertility and survival to the eyed stage of ova from fish maintained at 6,11 and 16°C (following temperature reduction to 8°C). Figures with the same superscripts are not significantly different ($P>0.05$). Values are mean ± SE.

3.3.ii Plasma Steroids

Due to the requirement by repeated measures ANOVA for orthogonal data, analysis could only be conducted up until day 35 of the study. Thereafter, removal of ovulated fish from the experiment prevented further analysis using this method. Hence, during the first six weeks of the study, mean plasma levels of each of the three steroids measured changed significantly with time ($P<0.001$, data not shown). However, only mean plasma E_2 displayed significant ($P<0.001$) temperature-related differences in the rate of that change (Figure 3.2). At all temperatures, mean plasma E_2 peaked after 7-14 days then declined to assay detection limits ($0.3 ng.ml^{-1}$) as ovulation progressed (Figure 3.2). Relative to 6 and 11°C, mean plasma E_2 was significantly lower at 16°C on days 1,7 and 14 ($P<0.05$). Furthermore, on day 21, mean plasma E_2 in fish maintained at 16°C was significantly lower ($P<0.01$) than that at 6°C while the difference relative to mean plasma E_2 at 11°C approached significance ($P=0.056$). There were no significant temperature-related differences in mean plasma E_2 at subsequent sample points.

Plasma T levels tended to increase with the onset of ovulation and decline thereafter (Figure 3.3). At 6°C, plasma T peaked on days 14 and 35 then fell to levels near or below $5 ng.ml^{-1}$ on days 50 and 56 coincident with the completion of ovulation at that

temperature. In fish held at 11°C, plasma T peaked on day 50 then declined to a level below 12ng.ml^{-1} on day 63. Following temperature reduction to 8°C, plasma T in fish previously maintained at 16°C peaked on day 71 then declined to a level below 5ng.ml^{-1} on day 78. Plasma T levels were generally lower at 16°C than at 6 or 11°C at most sample times until post-ovulatory falls occurred at 6 and 11°C, and the pre-ovulatory rise in T occurred in the 16°C group, following temperature reduction.

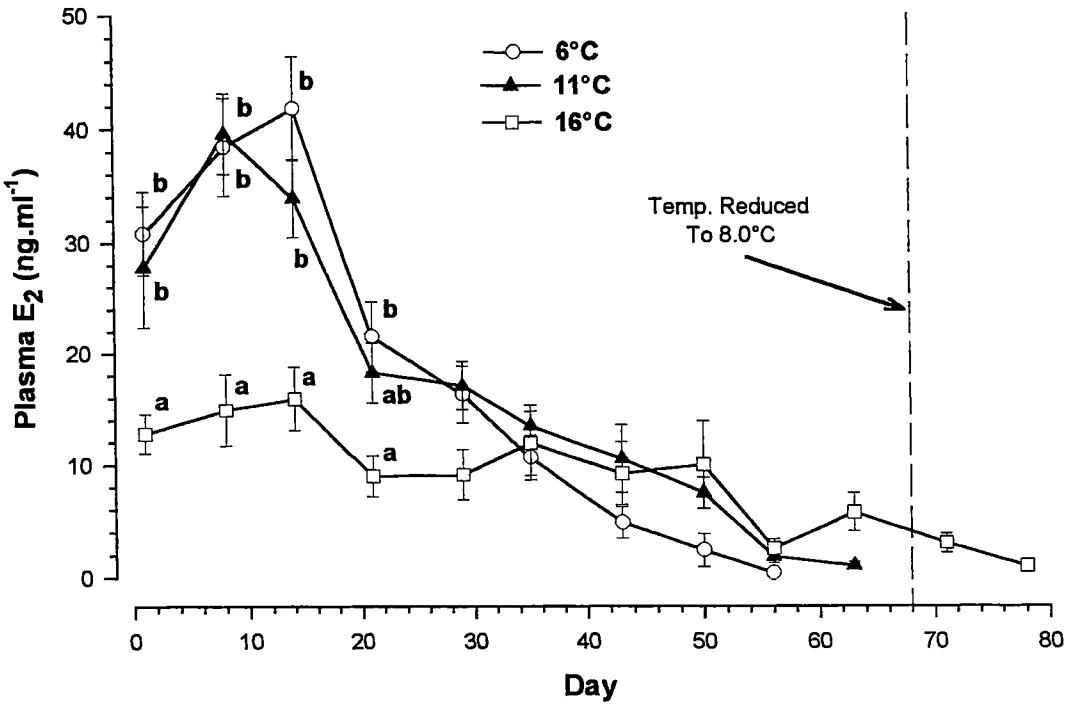

Figure 3.2 Plasma levels of E_2 in fish held at 6 (n=3-10), 11 (n=3-10) and 16°C (n=4-10). Data points with the same superscripts are not significantly different (P>0.05) at that time. Values are mean ± SE (non-significant test results have been omitted for clarity).

Plasma levels of 17,20βP in fish held at 6°C commenced at or below assay detection limits (0.3ng.ml^{-1}) and increased sharply prior to, and during ovulation, peaking on day 43 (Figure 3.4). From similar staring levels, an initial peak was observed on day 43 at 11°C, then 17,20βP declined coincident with the removal of ovulated animals from the experiment prior to the achievement of maximal levels on day 63 (late ovulating fish). At 16°C, 17,20βP remained at or below assay detection limits until

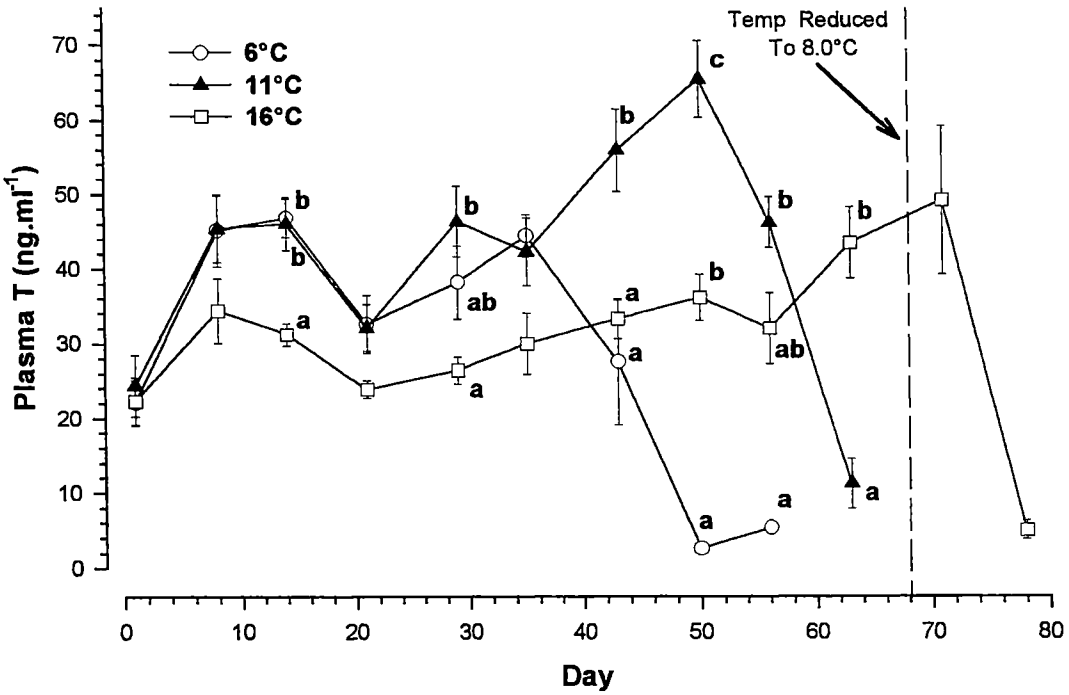

Figure 3.3 Plasma levels of T in fish held at 6 (n=3-10), 11 (n=3-10) and 16°C (n=4-10). Data points with the same superscripts are not significantly different (P>0.05) at that time. Values are mean ± SE (non-significant test results have been omitted for clarity).

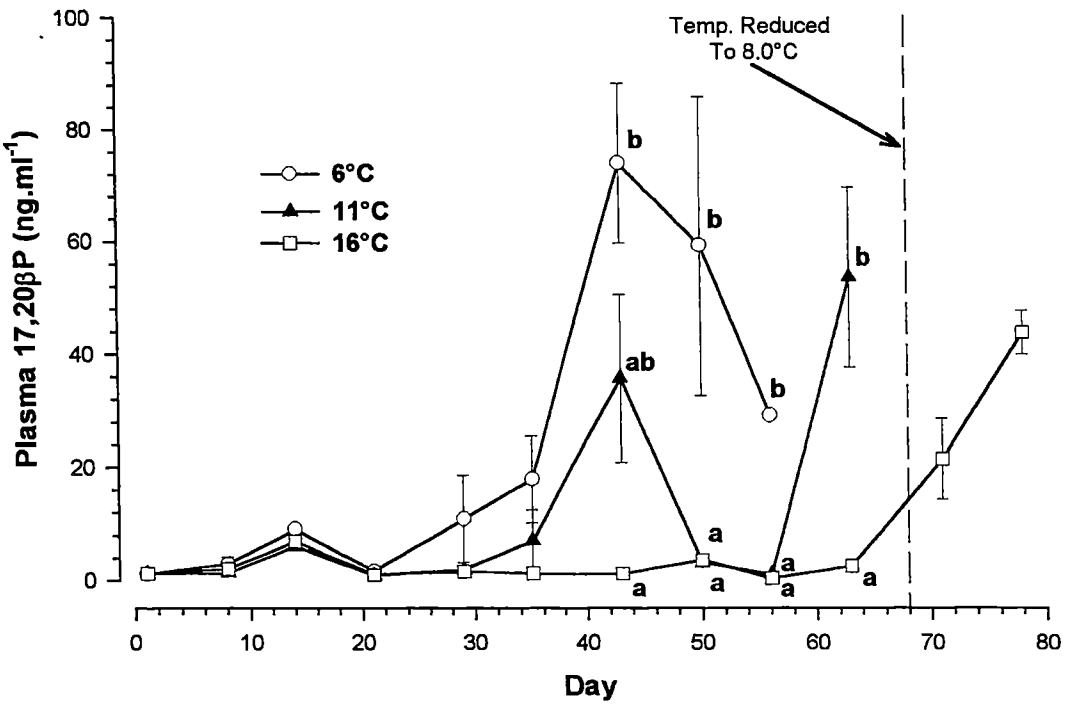

Figure 3.4 Plasma levels of 17,20βP in fish held at 6 (n=3-10), 11 (n=3-10) and 16°C (n=4-10). Data points with the same superscripts are not significantly different (P>0.05) at that time. Values are mean ± SE (non-significant test results have been omitted for clarity).

temperature reduction. Thereafter a maximal level of 17,20βP was observed on day 78 coincident with ovulation. While plasma 17,20βP levels tended to increase after day 21 at 6°C and after day 30 at 11°C, no significant temperature-related differences in 17,20βP were observed until day 43. Subsequently, plasma 17,20βP levels were generally higher at 6 and 11°C than at 16°C. However, on days 50 and 56, as a consequence of removal of the ovulated animals from the 11°C treatment group, plasma 17,20βP levels were not significantly different at 11 and 16°C.

3.4 Discussion

Ovulation in Atlantic salmon was delayed or inhibited by maintenance at elevated temperatures during late vitellogenesis and final oocyte maturation (FOM). Relative to fish held at 6°C, ovulation in fish held at 11°C was delayed (3-7 days) while ovulation in fish held at 16°C was inhibited until water temperature was reduced. These results are consistent with those reported in Arctic charr (Gillet, 1991), Atlantic salmon (Taranger and Hansen, 1993) and rainbow trout (Pankhurst et al., 1996). In Arctic charr from Lake Geneva, ovulation proceeded as normal at 5°C but was delayed at 8°C and inhibited at 11°C (Gillet, 1991). In a Norwegian stock of Atlantic salmon, ovulation initially proceeded as normal at 13-14°C but, after 2 weeks, ovulation declined and 22 out of 51 females failed to ovulate before the conclusion of the study. In contrast, all fish ovulated at 5-7°C and at natural temperatures (Taranger and Hansen, 1993). Similarly, in a Tasmanian stock of rainbow trout, ovulation was largely inhibited at 21°C, partially inhibited at 15 and 18°C and proceeded as normal at 9 and 12°C (Pankhurst et al., 1996).

Prior maintenance at 16°C was associated with a significant reduction in fertilisation relative to that of ova produced at 6 and 11°C. Furthermore, survival to the eyed stage was significantly lower in ova produced by fish maintained at 16°C relative to 11°C. Similar results were reported for each of the 3 species referred to above. Pankhurst et al. (1996) discussed the possibility that temperature shock and/or a temperature-related decline in the duration of post-ovulatory egg viability might contribute to the poor egg performance observed in those studies where there were relatively large differences between fish holding and egg incubation temperature regimes, and where ovulation did occur at higher temperatures. Pankhurst and

Thomas (1998) addressed the question of temperature shock and demonstrated that egg survival could be improved by incubating ova at the maternal holding temperatures of 12 and 18°C although normal larval development was impaired at the higher temperature. In the present study, however, ovulations only occurred at or below 11°C and females were held at 8°C for 24 hours prior to stripping and ova were incubated at 8°C. Therefore, the resulting maximum temperature differential of 3°C is considered unlikely to have influenced egg viability. Similarly, in view of the regularity of our ovulation checks, it is unlikely that reduced post-ovulatory egg viability was a significant factor. It is more likely that the observed differences in egg fertility and survival arose as a result of developmental abnormalities generated by maintenance at 16°C.

At 6°C, pre-ovulatory declines in plasma E_2 levels and peaks in plasma T and 17,20βP levels occurred in agreement with published steroid profiles for amago salmon (Young et al., 1983), coho salmon (Van Der Kraak et al., 1984; Estay et al., 1998), brook trout (Goetz et al., 1987), lake trout (Foster et al., 1993) and rainbow trout (Scott et al., 1982; Fostier et al., 1983; Pavlidis et al., 1994). In contrast to the observations of Pankhurst et al. (1996) in rainbow trout, where temperature effects on ovulation were not reflected in differences on plasma T or E_2; the inhibitory or delaying effects of maintenance at elevated temperatures on ovulation in Atlantic salmon were reflected in differing endocrine profiles. Relative to 6°C, there was no significant difference in the rate or extent of the pre-ovulatory decline in plasma E_2 levels in fish held at 11°C. However, consistent with delayed FOM and the observed delays in the commencement and completion of ovulation, pre-ovulatory peaks in plasma T and 17,20βP occurred later in fish held at 11°C than at 6°C. Plasma T and most notably, 17,20βP did not display significant peaks until after temperature reduction in fish maintained at 16°C, confirming temperature impairment of ovarian steroidogenesis, and in particular, the production of 17,20βP either as a result of a lack of pituitary gonadotropin release, or the incapacity of the granulosa cells of the ovarian follicle to synthesise 20β-hydroxysteroid dehydrogenase (20β-HSD).

During late vitellogenesis, fish held at 16°C displayed significantly reduced plasma E_2 levels relative to fish held at 6 and 11°C. Plasma T levels also tended to be lower in

fish held at 16°C, although the difference was significant on only one occasion. Pankhurst et al. (1996) noted broadly similar patterns of plasma E_2 and T in rainbow trout maintained at temperatures above 18°C, and although decreases in E_2 and T were not statistically significant, these workers cautioned that such a statistical outcome should be viewed with discretion. In the other studies where high temperatures were observed to inhibit or delay ovulation (Gillet, 1991; Taranger and Hansen, 1993) endocrine data were not collected, limiting the extent to which comparisons with the present study can be made; however, studies of wild coho salmon yield some useful observations. Flett et al. (1991, 1996) reported reduced ovulation, fertilisation and survival of ova in a Lake Erie stock of coho salmon, in association with lower levels of plasma T in pre-ovulatory animals, apparently as a result of exposure to elevated temperatures on the Pennsylvania shore of the lake. Viewed along with the data from the present study, these observations also tend to support impairment of ovarian steroidogenesis in fish held at high temperatures. Importantly, since the primary function of E_2 (and by association its precursor T) is the stimulation of vitellogenesis (hepatic synthesis and ovarian sequestration of the glycolipophosphoprotein vitellogenin; Specker and Sullivan, 1994), the above data also suggest that the reduced performance of ova from fish maintained at 16°C might result in part from impairment of vitellogenesis.

This assessment contrasts with that of Pankhurst et al. (1996) who reported that, despite an indication of reduced follicular steroidogenic capacity, female rainbow trout appeared to undergo normal vitellogenesis at high temperatures. Interestingly, in a later experiment, Pankhurst and Thomas (1998) observed smaller oocyte diameters at 18 than 12°C, possibly suggesting temperature effects prior to the maturational phase. Flett et al. (1996) also hypothesised that low fertility in wild coho salmon was related to a breakdown in the timing of FOM and/or ovulation. Paradoxically, Flett et al. (1996) presented data showing that, relative to Canadian stocks, American stocks of Lake Erie coho salmon experienced elevated temperatures primarily in late August and early September (1-3 months prior to ovulation) and reported that eggs appeared to deteriorate prior to entrance to the natal streams. Furthermore, these authors also reported retention of overripe ova in the ovarian stromal tissue while suggesting that American stocks suffered from excessively early ovulation as a result of exposure to

higher temperatures. These observations are somewhat confusing in that, retention of ova would tend to indicate at least a partial failure to ovulate rather than early ovulation. Further investigation is required to clarify the effect of elevated temperature on E_2 and T production, and vitellogenesis in salmonids.

Regardless of the exact nature of the physiological mechanisms involved, the present and previous studies in this, and other species clearly demonstrate the inhibitory effects of maintenance at elevated temperatures on ovulation and egg survival in a range of salmonids. As a consequence, it is reasonable to expect that phenomena such as possible global climate change and/or ENSO events are likely to have serious implications for the future sustainability of Atlantic salmon culture, particularly in those regions where natural temperature profiles are already marginal. Similarly, elevated summer and autumn water temperatures will exacerbate already recognised restrictions in off-season egg production achieved following photoperiod manipulation of Atlantic salmon (eg., Taranger and Hansen, 1993; Bromage, personal communication). In this regard, the observation that ovulation in heat-exposed fish could be restored, albeit with reduced egg viability, by short-term temperature reduction (all surviving fish maintained at 16°C ovulated within 7-10 days of water temperature reaching 8°C) suggests an avenue for further investigation in the search for potential remedial measures.

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CHAPTER 4

EFFECT OF MAINTENANCE AT ELEVATED TEMPERATURES ON OVULATION AND LHRHA RESPONSIVENESS IN TASMANIAN FEMALE ATLANTIC SALMON (*SALMO SALAR*)

4 EFFECT OF MAINTENANCE AT ELEVATED TEMPERATURES ON OVULATION AND LHRHA RESPONSIVENESS IN TASMANIAN FEMALE ATLANTIC SALMON (*SALMO SALAR*).

4.1 Introduction

Temperature is acknowledged as an important environmental parameter affecting the reproductive development and the timing of mating (spawning) in fish (reviewed by Van Der Kraak and Pankhurst, 1997). In salmonids in particular, both low and high temperatures have been observed to restrict or inhibit aspects of reproductive development (see Billard, 1985). In this context, a number of studies have demonstrated inhibition or delay of ovulation in salmonids in response to exposure to elevated temperatures (eg. Gillet, 1991; Taranger and Hansen, 1993; Pankhurst et al., 1996; Pankhurst and Thomas, 1998).

Our previous data on the ovulatory and endocrine responses of Tasmanian Atlantic salmon (*Salmo salar*)(Chapter 3) were consistent with the above observations. Because Tasmanian water temperatures approach the upper limits of the species' recognised thermal tolerance range those observations also indicated the susceptibility of Tasmanian Atlantic salmon stocks to any further increases in environmental temperatures (eg. from global climate change). Furthermore, as discussed by Taranger and Hansen (1993), Duncan et al. (2000) and Taranger et al. (2000), such data also indicate that normal late summer and early autumn elevations in temperature, such as those encountered in Tasmanian waters, are likely to constrain attempts to achieve a forward phase-shift in the reproduction of cultured stocks through photoperiod manipulation. In this regard, application of luteinizing hormone releasing hormone analogues (LHRHa) is known to promote and synchronise ovulation in Atlantic salmon (eg. Crim et al., 1983, 1986; Taranger et al., 1992) at normal spawning temperatures (eg. 3-10°C) and may represent a means for the restoration of ovulation in Atlantic salmon exposed to elevated temperatures either as a result of climatic events or phase-shifting of the reproductive cycle. However, the likely endocrine responses of Atlantic salmon to LHRHa treatment at elevated temperature are unclear. Numerous studies have examined the responsiveness of fish reproductive

endocrine homeostasis to temperature change, but most of those studies have been conducted on species such as the goldfish (*Carassius auratus*), the carp (*Cyprinus carpio*) and tilapias (*Oreochromis* sp.) (Van Der Kraak and Pankhurst, 1997). The validity of applying data derived from these species to salmonids is limited since many aspects of the reproductive endocrinology of salmonids (eg. gonadal steroid production) have come to be regarded as atypical of teleosts in general (Kime, 1993) As a consequence, endocrine data with direct relevance for any study of elevated temperature and ovulation in Atlantic salmon are essentially restricted to those of Pankhurst et al. (1996) and Pankhurst and Thomas (1998). In this context, it is noteworthy that Pankhurst and Thomas (1998) reported that, in addition to delaying the ovulation of control animals, exposure to elevated temperatures (18°C cf 12°C) resulted in delays in both the endocrine and ovulatory responsiveness of female rainbow trout (*Oncorhynchus mykiss*) to LHRHa injection. Production of the maturation inducing steroid 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) was delayed until administration of a second LHRHa treatment and ovarian follicles appeared to fail to express 20β-hydroxysteroid dehydrogenase (20β-HSD) in response to gonadotropin (GtH) administration *in vitro* (Pankhurst and Thomas, 1998).

The present study was conducted to examine the effect of maintenance at a range of water temperatures (6, 11 and 16°C) on the responsiveness of female Atlantic salmon to LHRHa. Having previously observed that ovulation was delayed and inhibited in fish held at 11°C and 16°C respectively (Chapter 3), and taking into account the observations of Pankhurst and Thomas (1998) of a delayed response to LHRHa in rainbow trout maintained at elevated temperatures, a primary objective of the study was to ascertain whether the endocrine function of fish held at 16°C was intact and capable of responding to LHRHa challenge. In this regard, Crim et al. (1988) examined changes in plasma GtH levels in testosterone-primed rainbow trout following LHRHa administration. A single liquid injection of LHRHa resulted in elevation of plasma GtH above basal levels for up to ~ 48 hours, but use of a cholesterol pellet as a vehicle for LHRHa increased the duration of GtH elevation from days to weeks (Crim et al., 1988). Consequently, in order to ensure that duration of GtH elevation was not limiting, fish were treated with LHRHa in saline solution or

in cholesterol pellets. Blood samples were collected at 48h intervals for up to 8 days post treatment and plasma was analysed for levels of the gonadal steroids testosterone (T), 17β-estradiol (E_2) and 17,20βP. Fertility and survival of ova were also assessed following regular ovulation checks.

4.2 Materials & Methods

4.2.i Stock, Husbandry, Temperature and Photoperiod Regimes

SALTAS broodstock (sexually maturing 2+ Atlantic salmon) were transported from Aquatas Pty. Ltd., (Margate, Tasmania) to SALTAS Freshwater Operations (Wayatinah, Tasmania) in late March 1997 and were initially maintained in outdoor concrete raceways supplied with river water at natural temperature (11-15°C). After 10 days, 80 female fish (mean weight 5.9kg) were transferred from natural conditions to groups of temperature-controlled $4m^3$ Rathbun tanks (10 fish per tank) set at final temperatures of 6, 11 and 16°C. Tanks were supplied with partially recirculated water. Biological filtration and water exchanges of 50% per day were employed to prevent the accumulation of toxic metabolites. Oxygen saturation was maintained at 100-120% by the addition of gaseous oxygen via ceramic diffusers. Transfer from natural temperature to 6°C was graded over 5 days. After 60 days the temperature in the 16°C system was reduced by $1°C.day^{-1}$ until a final water temperature of 8°C was achieved. Fish were exposed to a simulated natural photoperiod (42° S) for the duration of the study.

4.2.ii Treatments

35 days after transfer to the temperature-controlled systems, all fish from each of the three temperatures were anaesthetised (25p.p.m. benzocaine), weighed (± 0.1kg) and tagged by placing visible implant tags (VI Tags, Northwest Marine Technology Inc, Shaw Island, WA) in the left adipose eyelid according to the methods described by Bergman et al. (1992) and Kincaid and Calkins (1992). Blood samples were also taken from each fish by puncture of the duct of Cuvier (Lied et al., 1975) using heparinized (lithium heparin) syringes and 22G needles. After centrifugation, the resulting plasma was stored at -20°C prior to analysis of steroid levels. In addition,

the fish maintained at 11 and 16°C were randomly assigned to one of three treatment groups and either injected i.p. with saline and a blank cholesterol pellet (Control) or with a solution of des-Gly^{10}[D-Ala^{6}]-luteinizing hormone releasing hormone ethylamide (LHRHa, Sigma) in saline and a blank cholesterol pellet (LHRHa Injection), or with saline and a cholesterol pellet containing LHRHa (LHRHa Pellet). As fish were individually marked hormone treatments were mixed within holding tanks in order to eliminate tank effects. In fish maintained at 6°C, treatment with saline and an LHRHa pellet was omitted due to the commencement of spontaneous ovulations. Injection volume was 500µl.kg^{-1} body weight and dose for both LHRHa treatments was 25 µg.kg^{-1} body weight. Anaesthesia and blood sampling was repeated at 48 hour intervals until 8 days post injection. Ovulation checks were conducted at similar intervals and continued until all surviving fish had ovulated.

4.2.iii Ova Fertilisation and Incubation

Fish that expressed ova in response to the gentle application of pressure to the abdomen were transferred to a holding tank maintained at 8°C. After 24h at 8°C, fish were killed by a blow to the head, towelled dry and ova were expressed into a stainless steel sieve. In order to facilitate ova collection, a 2cm cut was made at the genital papilla. Ova were transferred to a stainless steel bowl and fertilised using pooled milt from 3-4 naturally spermiating males. Ova and milt were gently mixed and water (500ml) was added to ensure sperm activation. After 2 mins, ova were rinsed with clean water, then left to water-harden for 60 mins. After water-hardening sample batches (1000-1500) of ova from each female were removed and incubated at 8°C in mesh baskets (4.0cm x 10.0cm x 12.0cm) placed in Heath vertical incubator trays (Marisource Inc., Tacoma, WA). Sub-samples of ova from each female were collected after water-hardening, and fertilisation (%) was determined on the basis of first cell division after 120 degree-hours, visualised by treatment with a clearing solution (1:1:1 v/v methanol:acetic acid:water) for 2 mins. Eyed ova survival (%) was observed directly after 250 degree-days

4.2.iv Hormone Measurement

Plasma levels of E_2, T and 17,20βP were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst

and Carragher (1992). Extraction efficiency (mean recovery of ^{3}H-labelled steroid from triplicates of a plasma pool) ranged from 63-81, 75-87 and 91-100% for E_2, T and 17,20βP respectively, and assay values for each steroid were adjusted accordingly. Interassay variability measured using aliquots of a pooled internal standard was (%CV(n)) 10.2(12), 13.5(12) and 16.3(12) for E_2, T and 17,20βP respectively.

4.2.v Statistical Analysis

Where appropriate, data were analysed by one-way and two-way ANOVA and Tukey's HSD tests using the SYSTAT 8.0 for Windows computer package. However, due to the requirement by repeated measures ANOVA for a full orthogonal design with balanced sample numbers (Potvin et al., 1990), mortality and removal of ovulated fish from the experiment precluded analysis of hormone data using this method. Similarly, the removal of ovulated fish and the absence of a group of fish treated with saline and a cholesterol pellet containing LHRHa at 6°C hindered the application of two-way ANOVA to hormone data. As a consequence, the occurrence of treatment-related differences in plasma hormone levels over the course of the study was evaluated using an analysis of residual sums of squares (ARSS) according to the methods of Chen et al. (1992). The procedure for the comparison of pairs of treatments using the ARSS was as follows: (1) a second-order polynomial function was fitted to the data for each treatment, (2) residual sum of squares (RSS) and associated degrees of freedom (DF) were calculated for each treatment then summed to yield the total RSS and DF, (3) data for both treatments were pooled to calculate the RSS and DF of a single polynomial function fitted to the combined data, and (4) an *F*-statistic was calculated as

$$F = \frac{\dfrac{RSS_p - RSS_s}{DF_{RSS_p} - DF_{RSS_s}}}{\dfrac{RSS_s}{DF_{RSS_s}}} = \frac{\dfrac{RSS_p - RSS_s}{3(K-1)}}{\dfrac{RSS_s}{N-3K}}$$

where RSS_p = RSS of the polynomial function fitted to pooled data, RSS_s = sum of the separate RSS of the functions fitted to the data from each treatment, K = the number of treatments in the comparison (ie. 2), and N = total sample size. The

calculated F value was then compared with the critical F, with DFs of 3(K - 1) and N - 3K.

In spite of the assertion by Potvin et al. (1990) that such a curve-fitting approach to the analysis of repeated measures data is distribution free, the robustness of the theoretical F-distribution in the above analysis was confirmed using a randomization procedure. Sample data were repeatedly randomized (a total of 5,000 iterations were conducted) and a series of new F values were calculated as described above The new F values were then used to generate an empirical frequency distribution of the sampling statistic which was in turn, compared to the theoretical distribution in order to assess the possible extent of any deviations arising from the correlation existing among the data.

As a further safeguard against Type I error, pairwise comparisons were only made between temperature regimes within hormone treatment regimes, and between hormone treatment regimes within temperature regimes such that only 14 of a possible 28 pairwise comparisons were made.

Finally, proportion data were normalised by arcsin transformation.

4.3 Results

4.3.i Ovulation

In sham-injected controls (blank pellet plus saline), ovulations commenced first in fish maintained at 6°C where the first ovulation occurred 2 days post treatment and all fish ovulated within 12 days of treatment (Figure 4.1a). In contrast, in fish maintained at 11°C, ovulations did not commence until 6 days post treatment and, while 70% of fish had ovulated within 19 days, 100% ovulation did not occur until 36 days post treatment. At 16°C, there were no ovulations prior to temperature reduction. However, all six surviving fish ovulated within 10 days of temperature reduction. In fish treated with a blank pellet in combination with an LHRHa injection, ovulations also commenced first in fish held at 6°C (2 days post treatment) and this group completed ovulation on day 8 (Figure 4.1b). Similarly, the second group to commence

ovulation was that maintained at 11°C. Here ovulations commenced on day 4 and were completed 2 days later on day 6 post treatment. As observed in the sham-injected controls, ovulation was inhibited in fish held at 16°C with the 2 surviving animals completing ovulation within 10 days of temperature reduction. Of the two groups of fish which received an LHRHa pellet along with a saline injection, only those held at 11°C ovulated (Figure 4.1c). Ovulations in this group commenced on day 4 and were completed on day 13, with 80% of ovulations occurring by day 6 post treatment. None of the fish maintained at 16°C survived beyond day 33 and therefore the effect of temperature reduction on ovulation in this group could not be assessed.

4.3.ii Ova Fertility and Survival

In sham-injected controls, the fertility of ova from fish maintained at 16°C was significantly lower than that of ova from fish held at 6 and 11°C ($P < 0.001$ and $P < 0.01$ respectively, Figure 4.2a), whereas fertilities at 6 and 11°C were not significantly different. Similarly, the fertility of ova from fish held at 6 and 11°C and which had received an LHRHa injection did not differ significantly (Figure 4.2b) whereas the two surviving similarly treated fish held at 16°C failed to yield any viable ova. Fish maintained at 11°C and which received an LHRHa pellet yielded ova which exhibited a mean fertility similar to that observed in both other treatments at that temperature (Figure 4.2c). Within hormone treatment regimes, there were no temperature-related differences in eyed ova survival (Figure 4.3a, b and c). However, in sham-injected controls, eyed ova survival did display a marked tendency to decline with increasing holding temperature (Figure 4.3a) such that the difference in survival of ova from fish held at 6 and 16°C approached significance ($P = 0.061$).

Ova were not maintained beyond 250 degree-days.

4.3.iii Hormones

At all temperatures and in all treatments, mean plasma E_2 levels declined over the sampling period (Figure 4.4). Within treatments, there were no significant temperature-related differences in the pattern of that decline except in the groups which had received an LHRHa injection (Figure 4.4b; Table 4.1). Here, there was a

difference between fish at 11°C and those at 6 and 16°C due to the presence of high starting E_2 levels in fish held at 11°C relative to those at 6 and 16°C.

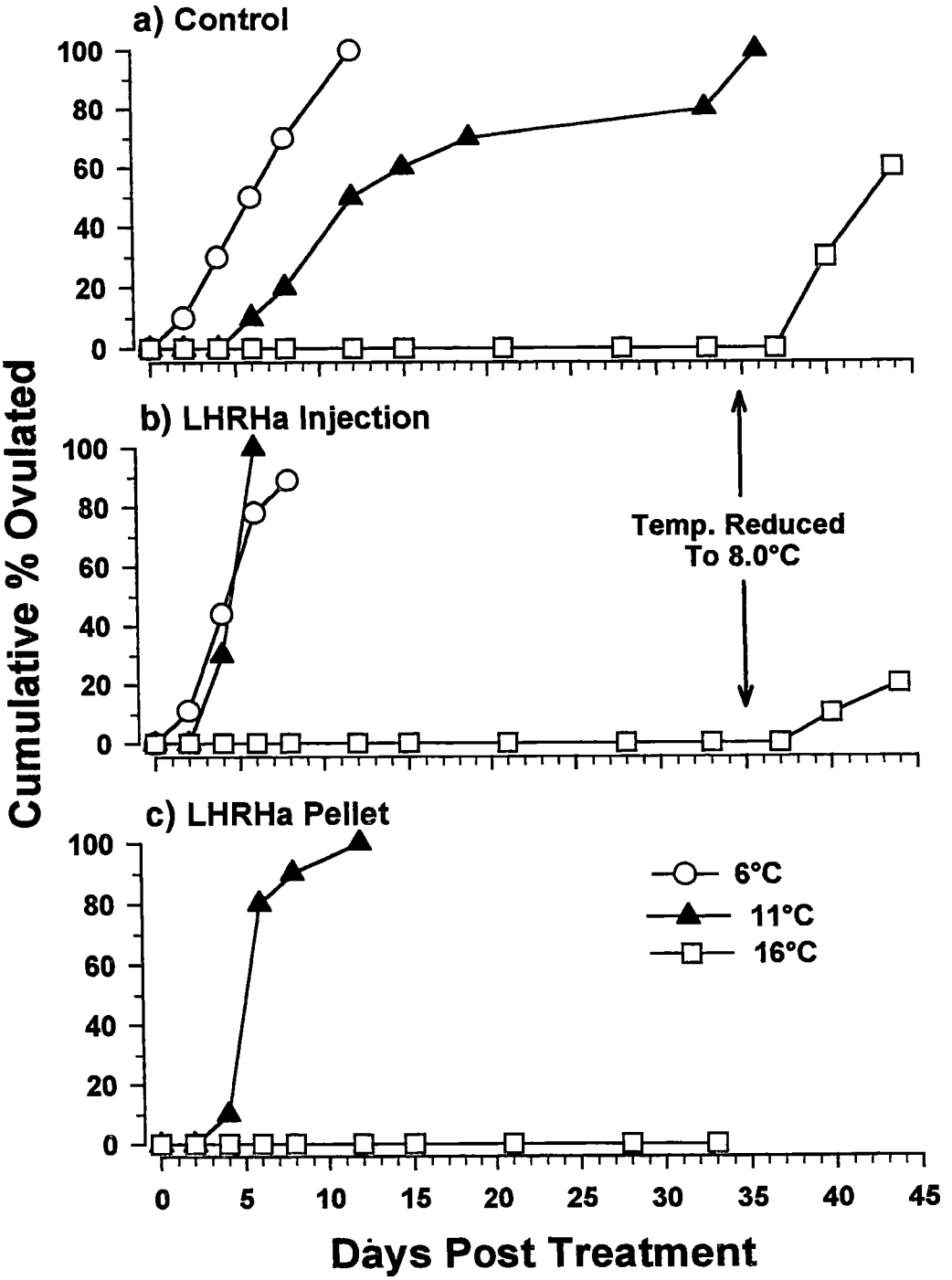

Figure 4.1 Cumulative percent ovulation in female Atlantic salmon maintained at 6 (○), 11 (▲) and 16°C (□) and treated with **a)** saline injection and a blank cholesterol pellet, **b)** LHRHa injection and blank pellet, and **c)** saline injection and an LHRHa pellet.

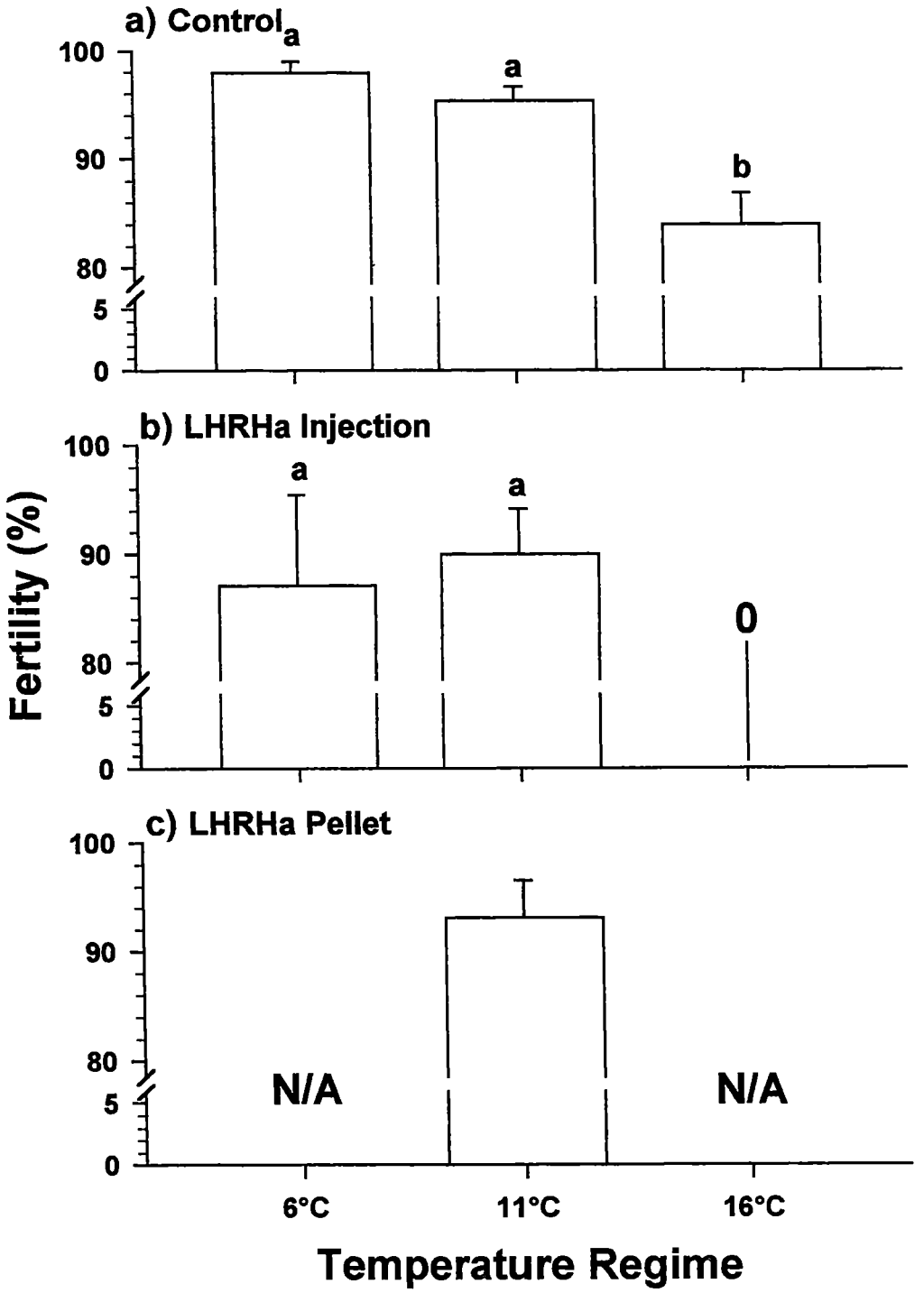

Figure 4.2 Mean (+ S.E.M., n = 9-10) percent fertility of ova from female Atlantic salmon maintained at 6, 11 and 16°C and treated with **a)** saline injection and a blank cholesterol pellet, **b)** LHRHa injection and blank pellet, and **c)** saline injection and an LHRHa pellet. Bars with the same superscript are not significantly different (P>0.05) (N/A = not available due to the absence of a treatment or the absence of viable ova).

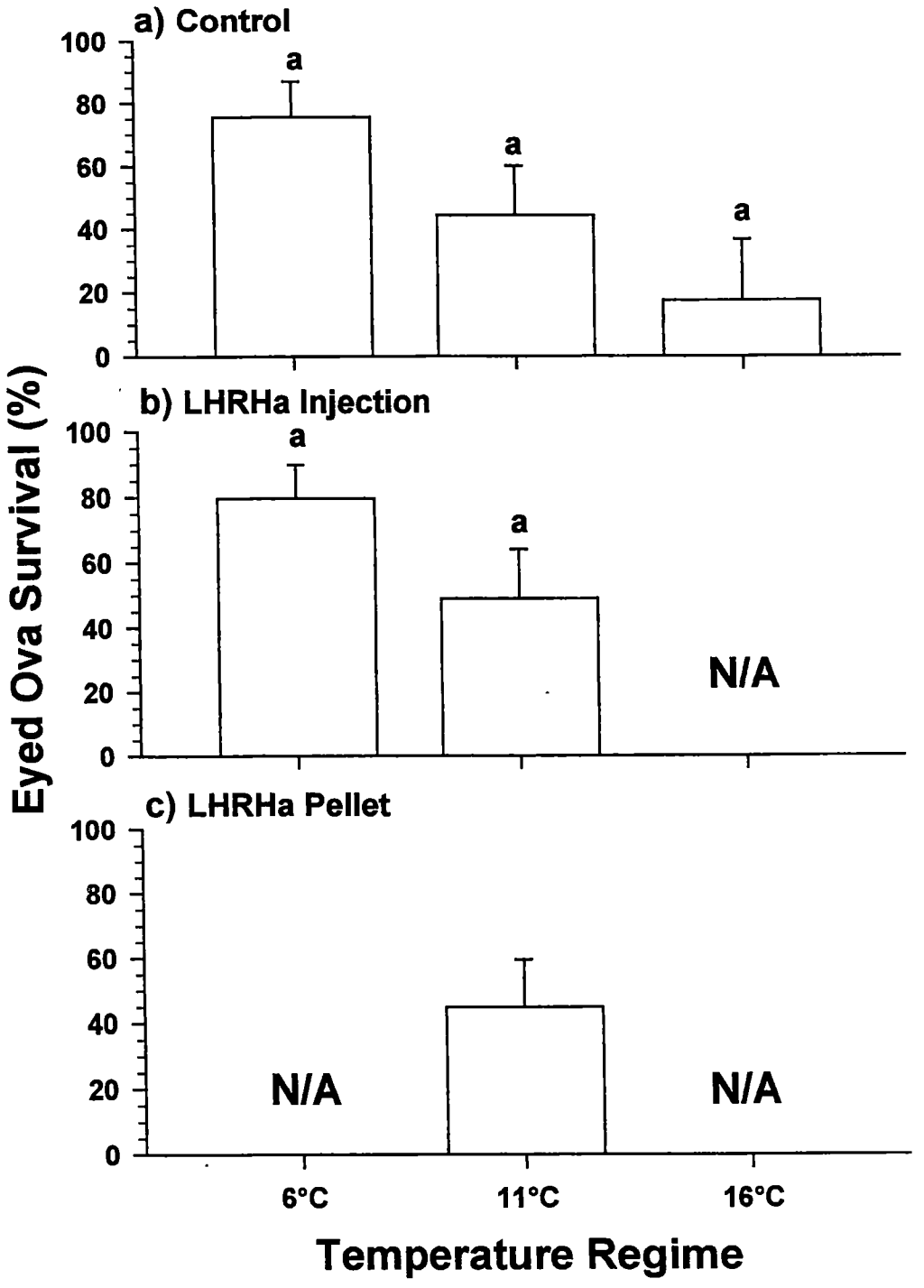

Figure 4.3 Mean (+ S.E.M., n = 9-10) percent survival to the eyed stage of ova from female Atlantic salmon maintained at 6, 11 and 16°C and treated with **a)** saline injection and a blank cholesterol pellet, **b)** LHRHa injection and blank pellet, and **c)** saline injection and an LHRHa pellet. Bars with the same superscript are not significantly different (P>0.05) (N/A = not available due to the absence of a treatment or the absence of viable ova).

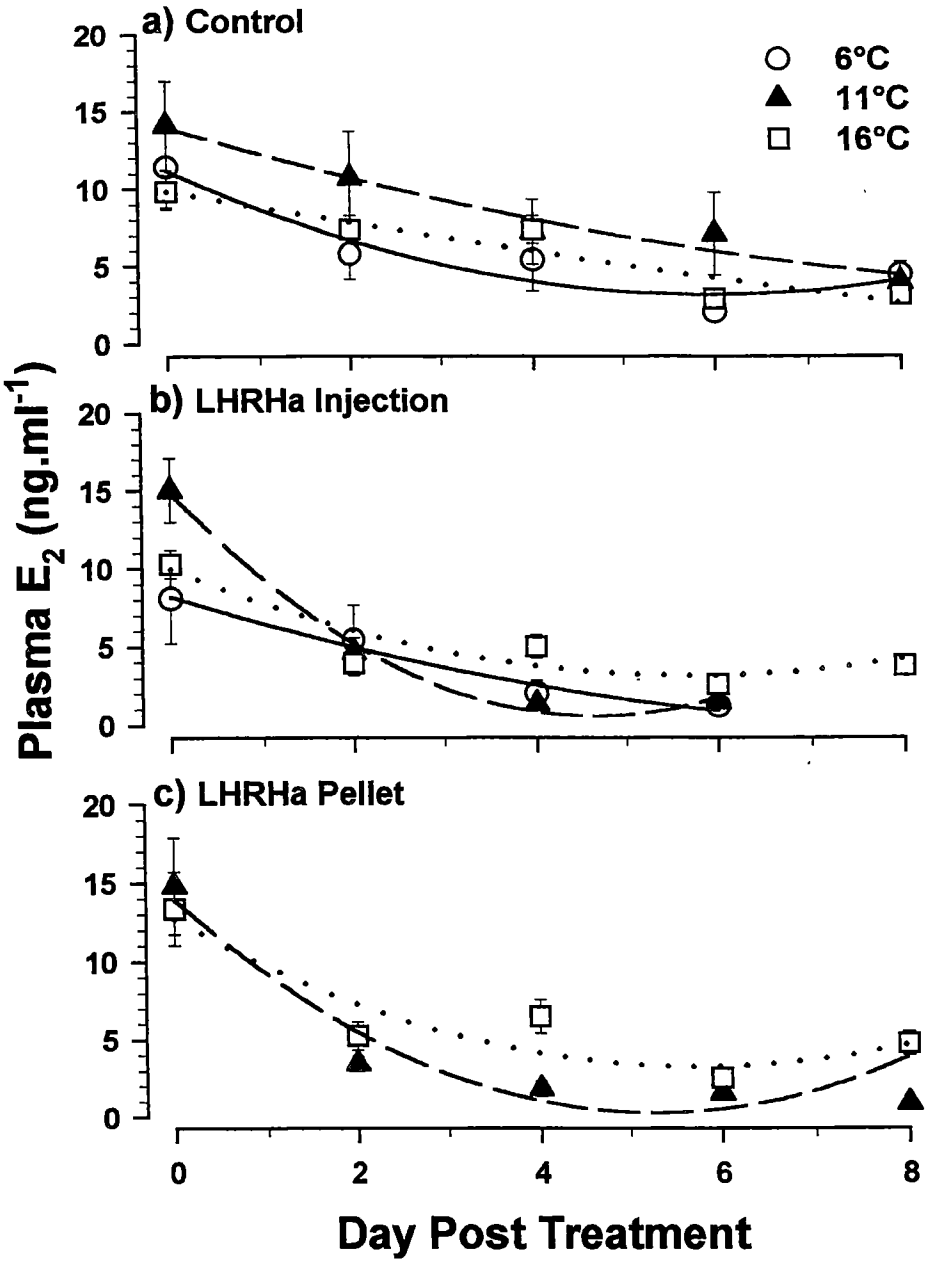

Figure 4.4 Mean (± S.E.M., n = 3-10) plasma 17β-estradiol (E_2 ng.ml^{-1}) in female Atlantic salmon maintained at 6 (○), 11 (▲) and 16°C (□) and treated with **a)** saline injection and a blank cholesterol pellet, **b)** LHRHa injection and blank pellet, and **c)** saline injection and an LHRHa pellet. Lines represent best fit 2nd order polynomial functions at 6 (—), 11 (- -) and 16°C (· ·). Outcomes of statistical comparisons are presented in Tables 1 and 2.

Table 4.1 Matrix of probabilities for pairwise within-treatment regime comparisons of plasma levels of E_2, T and 17,20βP in female Atlantic salmon held at 6, 11 or 16°C. Associated F-values and degrees of freedom are displayed in parentheses.

Steroid	Temperature (°C)	Treatment Regime: Control 11	Control 16	LHRHa Injection 11	LHRHa Injection 16	LRHa Pellet 11	LRHa Pellet 16
E2	**6**	**0.1872** ($F_{3,84}$ = 1.64)	**0.5201** ($F_{3,79}$ = 0.76)	**0.0315** ($F_{3,59}$ = 3.15)	**0.6556** ($F_{3,61}$ = 0.54)	**N/A**	**N/A**
	11	-	**0.1833** ($F_{3,87}$ = 1.65)	-	**0.0015** ($F_{3,70}$ = 5.69)	-	**0.1820** ($F_{3,83}$ = 1.66)
T	**6**	**0.0054** ($F_{3,84}$ = 4.53)	**0.0012** ($F_{3,79}$ = 5.85)	**0.0361** ($F_{3,59}$ = 3.04)	**0.0195** ($F_{3,61}$ = 3.55)	**N/A**	**N/A**
	11	-	**0.3328** ($F_{3,87}$ = 1.15)	-	**<0.0001** ($F_{3,70}$ = 8.96)	-	**0.0001** ($F_{3,83}$ = 7.80)
17,20βP	**6**	**<0.0001** ($F_{3,84}$ = 24.71)	**<0.0001** ($F_{3,79}$ = 35.40)	**0.0007** ($F_{3,59}$ = 6.56)	**<0.0001** ($F_{3,61}$ = 61.98)	**N/A**	**N/A**
	11	-	**0.0002** ($F_{3,87}$ = 7.17)	-	**<0.0001** ($F_{3,70}$ = 18.83)	-	**<0.0001** ($F_{3,83}$ = 35.71)

Comparison of treatments within temperature regimes showed that there were no significant treatment-related differences in the patterns of E_2 production at 6°C (Table 4.2), whereas, at 11°C, treatment with LHRHa in both liquid and solid carriers resulted in patterns of decline (Figure 4.4b and c) which differed significantly from that observed in sham-injected controls (P=0.0151 and P=0.0162 respectively, Table 4.2). Similarly, at 16°C, treatment with a blank pellet and an LHRHa injection resulted in E_2 production which differed from that of sham-injected controls (P=0.0146, Table 4.2).

Rates of changes in mean plasma T levels varied with both temperature and treatment (Figure 4.5). In sham-injected controls (Figure 4.5a), mean plasma T of fish held at 11 and 16°C T tended to remain above 20ng.ml^{-1} whereas at 6°C, plasma T levels declined with time. As a consequence, plasma T at 6°C differed significantly from that observed at 16°C (P=0.0012, Table 4.1) and 11°C (P=0.0054, Table 4.1). Similarly, in fish which had received an LHRHa injection (Figure 4.5b) plasma T did not decline at 16°C; differing significantly from the declining trends in fish held at 6 and 11°C (P=0.0195 and P<0.0001 respectively, Table 4.1). Here, as observed for plasma E_2, the difference was largely due to an elevated starting T level in fish held at 11°C. In LHRHa pellet-treated animals, plasma T also declined at 11°C whereas T production tended to increase in fish maintained at 16°C (Figure 4 5c). As a consequence, the temperature-related difference in pattern of T production in LHRHa pellet-treated fish was highly significant (P=0.0001, Table 4.1).

Within the 6°C temperature regime, mean plasma T levels tended to decline more rapidly in response to LHRHa injection but the difference relative to the declines observed in sham-injected controls and LHRHa pellet-treated animals was not significant. This trend was clearer at 11°C where LHRHa delivered in a liquid carrier, was associated with a pattern of T production which differed significantly from that of sham-treated controls (P<0.0001, Table 4.2). Similarly, LHRHa delivered via a solid carrier was associated with a pattern of T production which differed significantly from that of sham-treated controls (P=0.0044, Table 4.2).

Table 4.2 Matrix of probabilities for pairwise within-temperature regime comparisons of plasma levels of E_2, T and 17,20βP in female Atlantic salmon treated with LHRHa. Associated F-values and degrees of freedom are displayed in parentheses.

Steroid	Treatment	Temperature Regime					
		6°C		11°C		16°C	
		LHRHa Injection	LHRHa Pellet	LHRHa Injection	LHRHa Pellet	LHRHa Injection	LHRHa Pellet
E_2	Control	**0.5906** ($F_{3,58}$ = 0.64)	N/A	**0.0151** ($F_{3,71}$ = 3.73)	**0.0162** ($F_{3,72}$ = 3.66)	**0.0146** ($F_{3,85}$ = 3.71)	**0.1097** ($F_{3,86}$ = 2.07)
	LHRHa Injection	-	N/A	-	**0.9864** ($F_{3,70}$ = 0.05)	-	**0.1562** ($F_{3,89}$ = 1.78)
T	Control	**0.0889** ($F_{3,58}$ = 2.28)	N/A	**<0.0001** ($F_{3,71}$ = 10.87)	**0.0044** ($F_{3,72}$ = 4.75)	**0.1755** ($F_{3,85}$ = 1.69)	**<0.0001** ($F_{3,86}$ = 8.54)
	LHRHa Injection	-	N/A	-	**0.1742** ($F_{3,70}$ = 1.70)	-	**<0.0001** ($F_{3,89}$ = 9.03)
17,20βP	Control	**0.4483** ($F_{3,58}$ = 0.89)	N/A	**<0.0001** ($F_{3,71}$ = 11.99)	**<0.0001** ($F_{3,72}$ = 22.24)	**0.1333** ($F_{3,85}$ = 1.91)	**0.0109** ($F_{3,86}$ = 3.95)
	LHRHa Injection	-	N/A	-	**0.3390** ($F_{3,70}$ = 1.14)	-	**0.3289** ($F_{3,89}$ = 1.16)

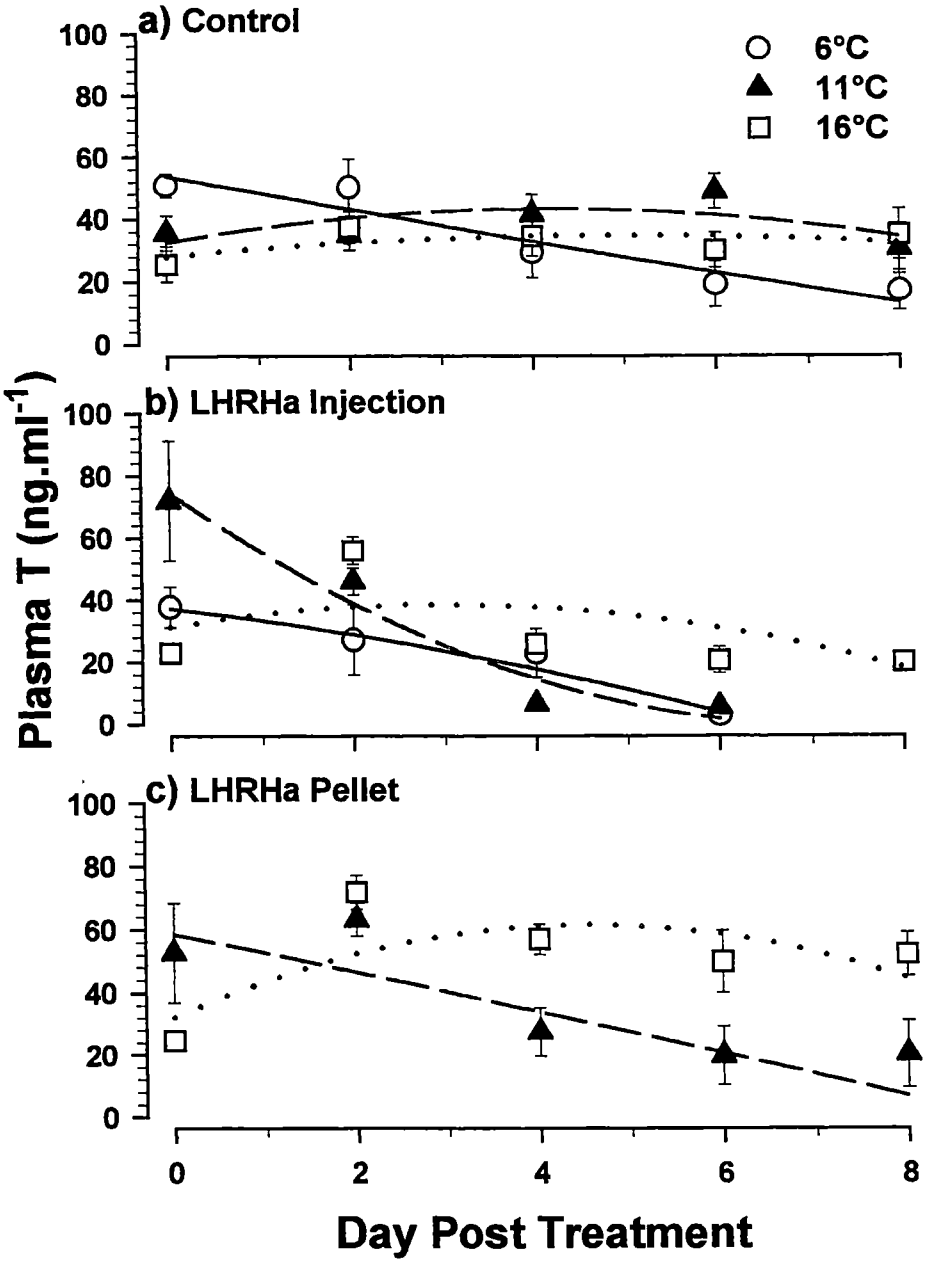

Figure 4.5 Mean (± S.E.M., n = 3-10) plasma testosterone (T ng.ml^{-1}) in female Atlantic salmon maintained at 6 (○), 11 (▲) and 16°C (□) and treated with **a)** saline injection and a blank cholesterol pellet, **b)** LHRHa injection and blank pellet, and **c)** saline injection and an LHRHa pellet. Lines represent best fit 2nd order polynomial functions at 6 (—), 11 (- -) and 16°C (· ·). Outcomes of statistical comparisons are presented in Tables 1 and 2.

In contrast, at 16°C, LHRHa pellet treatment was associated with an apparent increase in mean plasma T levels whereas, in the other treatments, T levels tended not to change over time. As a consequence highly significant differences were observed ($P<0.0001$, Table 4.2).

17,20βP production was evident on day 0 in fish held at 6°C, whereas plasma levels of 17,20βP in fish maintained at 11 and 16°C were at or below assay detection limits (Figure 4.6). Thereafter, 17,20βP production differed significantly between all three temperature regimes. In sham-injected controls (Figure 4.6a), high levels of 17,20βP were observed in fish maintained at 6°C (up to ~ 115 $ng.ml^{-1}$) which differed significantly ($P<0.0001$, Table 4.1) from those observed in fish held at 11°C (up to ~ 25 $ng.ml^{-1}$) and 16°C where levels of 17,20βP were barely detectable throughout.

17,20βP levels observed at 11°C were also significantly higher than those observed at 16°C ($P<0.0002$, Table 4.1). Similar results and levels of significance were observed in fish which received an LHRHa injection (Figure 4.6b, Table 1), whereas in LHRHa pellet-treated fish (Figure 4.6c), some additional production of 17,20βP occurred towards the end of sampling in animals held at 16°C (up to ~ 25 $ng.ml^{-1}$).

Nevertheless, production of 17,20βP at 16°C continued to differ significantly from that observed in fish held at 11°C ($P<0.0001$, Table 4.1).

Treatment-related differences in 17,20βP production were also observed within temperature regimes. Treatment with an LHRHa injection appeared to be associated with accelerated 17,20βP production at 6°C, however, there was no significant difference relative to controls ($P=0.4483$, Table 4.2). In contrast, at 11°C, 17,20βP production was significantly increased relative to that in sham injected controls, following either LHRHa treatment ($P<0.0001$, Table 4.2). There was no difference in response to LHRHa injection relative to LHRHa pellet treatment. Finally, at 16°C, the increase in production of 17,20βP which occurred towards the end of sampling in fish treated with an LHRHa pellet was significantly different from that of sham-injected controls ($P=0.0109$, Table 4.2).

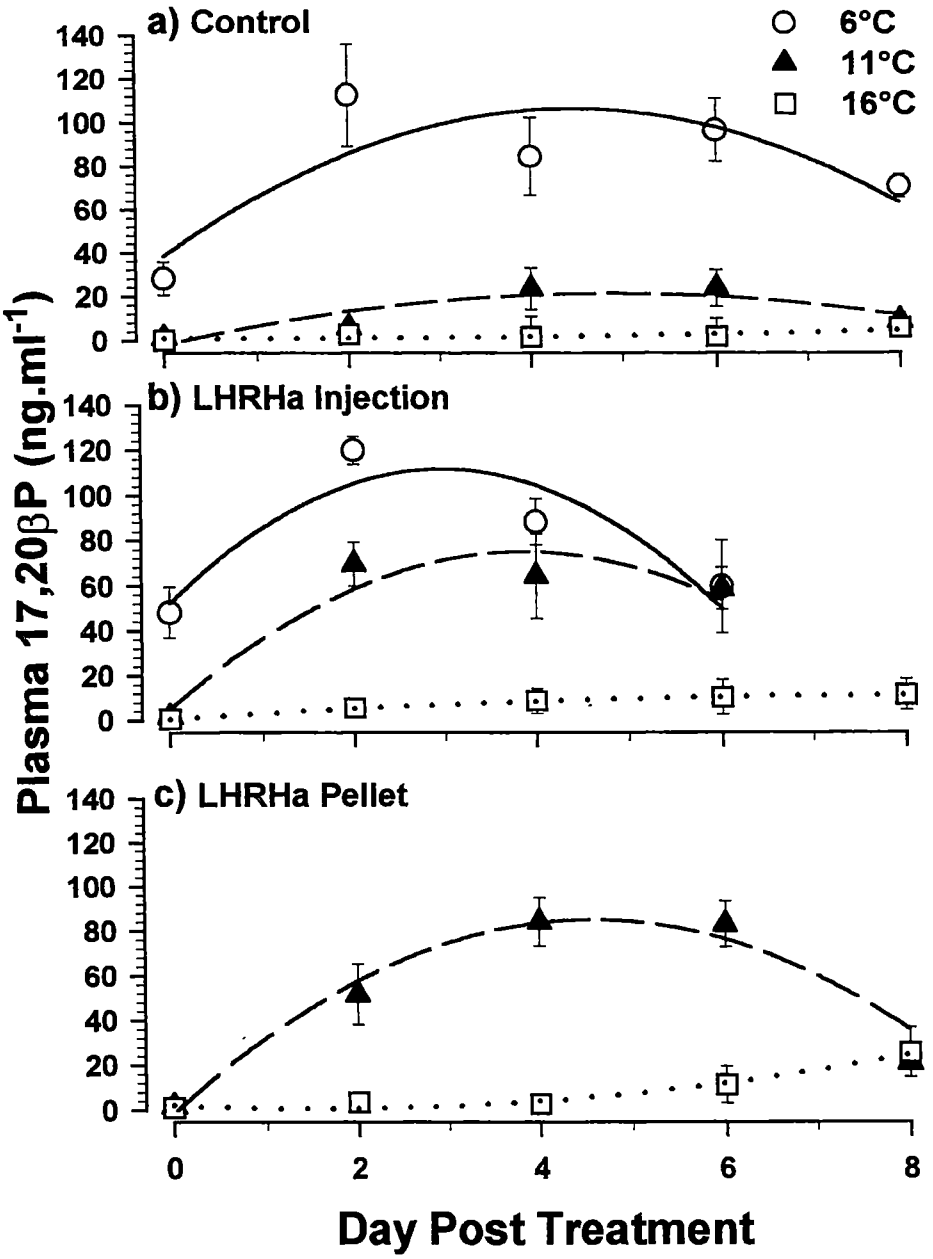

Figure 4.6 Mean (± S.E.M., n = 3-10) plasma 17,20β-dihydroxy-4-pregnen-3-one (17α,20βP ng.ml^{-1}) in female Atlantic salmon maintained at 6 (○), 11 (▲) and 16°C (□) and treated with **a)** saline injection and a blank cholesterol pellet, **b)** LHRHa injection and blank pellet, and **c)** saline injection and an LHRHa pellet. Lines represent best fit 2nd order polynomial functions at 6 (——), 11 (- -) and 16°C (· ·). Outcomes of statistical comparisons are presented in Tables 1 and 2.

4.4 Discussion

Consistent with the results of our previous study (Chapter 3), ovulation in sham-treated female Atlantic salmon was delayed or inhibited as a result of maintenance at elevated temperatures. There was a 4-24 day delay in ovulation at 11°C relative to 6°C and, as previously observed, ovulation was inhibited in fish held at 16°C prior to temperature reduction. As previously discussed, these results are consistent with those reported in Arctic charr (Gillet, 1991), Atlantic salmon (Taranger and Hansen, 1993) and rainbow trout (Pankhurst et al., 1996) as well as a non-salmonid; the common wolffish *Anarhichas lupus* L. (Tveiten et al., 2001) where maintenance at 8 and 12°C during the breeding season was associated with delayed ovulation relative to fish held at 4°C.

Fish maintained at 6°C exhibited accelerated and highly synchronised ovulation following LHRHa injection, as did fish held at 11°C following either LHRHa injection or pellet treatment. These observations are in agreement with those reported in a number of studies of Atlantic salmon (eg. Crim et al., 1893, 1986; Taranger et al., 1992) as well as other salmonids (eg. Donaldson et al., 1981,1985; Sower et al., 1984; Van Der Kraak et al., 1984,1985; Mylonas et al., 1992) and non-salmonids (see reviews by Peter et al., 1993 and Patiño, 1997). In particular, Taranger et al. (1992) using the same LHRH analogue as in the present study, observed a most consistent ovulatory response by female Atlantic salmon when LHRHa treatments were applied close to the normal time of ovulation in controls (approximately 14-28 days prior to median ovulation). In this regard, during the present study, 6 and 11°C control fish achieved 50% ovulation 6 and 12 days after treatment respectively. In contrast to the observations at lower temperatures, neither LHRHa treatment resulted in the ovulation of fish held at 16°C. Pankhurst and Thomas (1998) reported a similar outcome in rainbow trout maintained at 18°C. However, these authors found that fish did respond to a second LHRHa injection 25 days after first treatment, indicating that maintenance at elevated temperature delayed rather than inhibited the acquisition of LHRHa responsiveness in rainbow trout. During the present study, a second LHRHa treatment was not applied and as a result, it is unclear whether a similar retardation was occurring in Atlantic salmon or whether elevated temperature was acting to

inhibit LHRHa action in this species. Nevertheless, the fact that 16°C fish were maintained for 35 days with no ovulations, whereas Pankhurst and Thomas (1998) observed spontaneous ovulations in 18°C trout after 25 days, tends to favour the latter hypothesis.

The high fertility and survival of ova from fish maintained at 6°C and the consistency of levels of fertility and survival of ova from fish held at 6 and 11°C irrespective of LHRHa treatment are in agreement with the results of Taranger et al. (1992) and Haraldsson et al. (1993) where only high doses of LHRHa (100-150 $mg.kg^{-1}$) were associated with a significant reduction in the survival of Atlantic salmon ova to the eyed stage. Similarly, the trend of declining egg survival with increasing temperature is consistent with the observations of Pankhurst et al. (1996) and Pankhurst and Thomas (1998) where the survival of ova from rainbow trout held at 18°C was significantly lower than that of ova from fish held at 12°C while the survival of ova from fish held at 15°C was reduced relative to that at 12°C but not significantly so. Similar observations have been made in Arctic charr (Gillet, 1991) and Norwegian Atlantic salmon (Taranger and Hansen, 1993) while Tveiten et al. (2001) also observed temperature-related declines in the levels of eyed survival and hatching in wolffish ova. The significant reduction in the fertility of ova from sham-injected controls held at 16°C and the failure of LHRHa-injected fish held at 16°C to produce any viable ova are more difficult to comment upon as no fertility data were presented in the studies by Gillet (1991), Taranger and Hansen (1993) and Pankhurst et al. (1996), while Pankhurst and Thomas (1998) assessed fertility at a later stage (neural streak). Moreover, Tveiten et al. (2001) presented data showing no effect of temperature on fertilisation success in wolffish ova. However, Flett et al. (1996) did report highly variable fertility (ranging from 0 to greater than 80%) in a study of thermally compromised coho salmon (*Oncorhynchus kisutch*) from the Great Lakes of North America. Thus, the present results may reflect the fact that temperature influences the fertilisation capacity as well as the long-term survival of salmonid ova.

The preovulatory declines in the plasma levels of E_2 of control fish maintained at 6 and 11°C and the acceleration of those declines in LHRHa-treated fish during the present study are consistent with those observed in coho salmon held at 10°C (Van Der Kraak et al., 1984,1985), Atlantic salmon maintained at ambient temperatures

(Crim et al., 1986) and rainbow trout held at 12°C (Pankhurst and Thomas, 1998). In one study, Van Der Kraak et al. (1984) reported a significant decline in the plasma E_2 level of saline-injected fish 96h post injection while in two studies, the plasma E_2 levels of LHRHa-injected fish were significantly reduced relative to those of controls within 48-96h of treatment (Van Der Kraak et al., 1984,1985). Pankhurst and Thomas (1998) also reported a significant reduction relative to controls in the E_2 levels of LHRHa-treated rainbow trout at 72 and 96h post injection, while the fall in serum E_2 level in LHRHa-treated Atlantic salmon reported by Crim et al. (1986) commenced approximately 28 days earlier than that observed in controls. However, the present observation of similar declines in the plasma E_2 levels of fish held at 16°C and in particular, the slight but statistically significant acceleration of that decline in LHRHa-injected fish, conflicts with the observations of Pankhurst and Thomas (1998) where E_2 levels remained constant for up to 96h post injection in both saline- and LHRHa-injected rainbow trout maintained at 18°C. As previously discussed (Chapter 3), the low and declining plasma E_2 of fish held at 16°C is in broad agreement with the patterns observed in rainbow trout maintained at temperatures above 18°C (Pankhurst et al., 1996) and may reflect general impairment of ovarian steroidogenesis in fish exposed to elevated temperatures. The slight acceleration in the decline in E_2 levels in LHRHa-injected fish at 16°C tends to indicate some responsiveness to LHRHa at the steroidogenic level if not at the level of ovulation. The latter conclusion is supported by the observation of increased T production in LHRHa pellet-treated animals at 16°C. In this context, Pankhurst and Thomas (1998) also observed a significant increase in plasma T 48h post LHRHa injection in rainbow trout held at 18°C. Similar increases in plasma T have been observed at lower temperatures (eg. 10-12°C) in the range of species previously referred to (Van Der Kraak et al., 1894; Crim et al., 1986; Pankhurst and Thomas, 1998), although such increases tend only to be transient and are followed by significant declines which reflect the shift to progestin synthesis which characterises the recognised maturational endocrine profiles of salmonids (reviewed by Fostier et al., 1983 and Kime, 1993).

The decline in plasma T observed under the majority of treatments during the present study probably reflects the shift in steroidogenesis to production of the maturation-inducing steroid (MIS) 17,20βP while the maintenance or elevation of plasma T levels

and minimal production of 17,20βP in fish held at 16°C, indicates a lack of maturational competence consistent with their failure to ovulate prior to temperature reduction. In this regard, the high production of 17,20βP by control fish held at 6°C and its acceleration in response to LHRHa injection is also consistent with the recognised pattern of steroid production in naturally ovulating salmonids (reviewed by Fostier et al., 1983), and observations of its advancement in response to LHRHa administration in a variety of species (eg. Van Der Kraak et al., 1984,1985; Crim et al., 1986). Similarly, the relatively low levels of 17,20βP observed in sham-injected fish maintained at 11°C is consistent with our previous observations (Chapter 3) as well as the observed delay in ovulation, while the responsiveness of fish held at 11°C to LHRHa treatment provides confirmation that 11°C fish retained essentially normal endocrine function. In contrast, the lack of 17,20βP production in fish held at 16°C and in particular those treated with LHRHa, is indicative of endocrine dysfunction.

The absence of GtH data in the present study hinders interpretation of other endocrine data and as a result, it is unclear whether the 17,20βP levels observed at 16°C reflect inadequate pituitary responsiveness to LHRHa (in spite of the fact that T data indicate at least a modest response in LHRHa pellet-treated animals) or a lack of ovarian 20β-hydroxysteroid dehydrogenase (20β-HSD) activity. On the basis of similar data, Pankhurst and Thomas (1998) suggested that LHRHa did not strongly stimulate pituitary GtH release. However, these authors also presented *in vitro* data which indicated that, in oocytes from rainbow trout held at 18°C, aromatase activity and thus E_2 production was favoured over 20β-HSD activity and 17,20βP production Together with the results of the present study, these observations point to the conclusion that both inadequate pituitary responsiveness and a lack of 20β-HSD were responsible for the observed failure of heat-exposed fish to produce significant quantities of 17,20βP.

Irrespective of the mechanisms involved, the present success of LHRHa treatments in fish held at 11°C and their failure in fish held at 16°C also serve to indicate that LHRHa treatment is likely to be of only limited value in overcoming the temperature-related constraints on forward phase-shifting of spawning in cultured Atlantic salmon identified by a number of authors (Taranger and Hansen, 1993; Duncan et al., 2000;

Taranger et al., 2000). However, this and previous studies (Chapter 3; Pankhurst and Thomas, 1998) also suggest the potential for short-term temperature ramp-down as a means of restoring ovulation in heat-exposed fish. Furthermore, it may be possible to combine LHRHa treatment and temperature reduction to enhance the ovulatory response. Here, the present observation of the failure of LHRHa-injected fish to produce viable ova following temperature reduction should be viewed with caution. In this instance, 35 days had elapsed between LHRHa administration and temperature reduction and as a consequence, the present results are unlikely to accurately reflect the response of fish if treated at the time of temperature reduction.

Finally, it is interesting to note the differential survival of fish maintained at 16°C. Consistent with previous results in un-manipulated fish (Chapter 3), 7 out of 10 sham-treated animals survived to the end of the experiment. In contrast, only 2 LHRHa-injected animals survived to ovulate while no LHRHa pellet-treated fish survived beyond day 33 of the study. In view of the fact that these groups of fish underwent identical treatment and handling procedures, the reason for the differences in their survival is not immediately apparent. However, in this regard, Dickhoff (1989) reported that, relative to saline-injected controls or untreated animals, a captive broodstock of Atlantic salmon consistently exhibited high post-spawning mortality following LHRHa treatment. Typically, 10-40% of naturally spawned fish would recover to spawn repeatedly whereas, no LHRHa-treated animals survived for more than a month post spawning. Furthermore, in a controlled study, LHRHa treatment increased mortality in a dose-dependent manner while exposure of fish to elevated water temperatures (13-15°C) further increased mortality in LHRHa-treated fish (Dickhoff, 1989). As a result, it was concluded that these observations supported the role of the ovulatory surge in GtH-II as a primary endocrine agent in the mediation of programmed death in semelparous species (Dickhoff, 1989).

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CHAPTER 5

EFFECT OF SHORT-TERM TEMPERATURE REDUCTION ON OVULATION AND LHRHA RESPONSIVENESS IN TASMANIAN FEMALE ATLANTIC SALMON (*SALMO SALAR*)

5 EFFECT OF SHORT-TERM TEMPERATURE REDUCTION ON OVULATION AND LHRHA RESPONSIVENESS IN TASMANIAN FEMALE ATLANTIC SALMON (*SALMO SALAR*).

5.1 Introduction

Exposure to elevated temperatures during vitellogenesis and/or the periovulatory period can impair reproductive development in female salmonids. For example, in both the Atlantic salmon (*Salmo salar*) and the Arctic charr (*Salvelinus alpinus*), ovulation was delayed and/or partially inhibited in response to exposure to above-normal temperatures (Gillet, 1991; Taranger and Hansen, 1993; Jobling et al., 1995). However, as no endocrine data were presented during these studies, the likely mechanism or mechanisms of temperature impairment of ovulation could not be determined. In contrast, endocrine data relating to the responses of female rainbow trout (*Oncorhynchus mykiss*) to temperature elevation, indicated that expression of 20β-hydroxysteroid dehydrogenase (20β-HSD); the enzyme which catalyses the conversion of the steroid precursor 17α-hydroxyprogesterone (17P) to 17,20β-dihydroxy-4-pregnen-3-one (17,20βP: reviewed by Nagahama, 1997, 2000), was delayed at elevated temperatures (Pankhurst and Thomas, 1998). Since 17,20βP is the acknowledged maturation-inducing steroid (MIS) in salmonids (reviewed by Nagahama, 1997, 2000), this conclusion was consistent with the earlier work of Pankhurst et al. (1996) where it was determined that the effects of elevated temperature on trout reproductive development were restricted to events associated with final oocyte maturation (FOM).

Our own studies of the responses of female Atlantic salmon to maintenance at elevated temperatures (Chapter 3 and 4) generated results which were broadly similar to those outlined above and, in particular, those of Pankhurst et al. (1996) and Pankhurst and Thomas (1998). In untreated or sham-treated animals in the present studies, ovulation was delayed at intermediate temperatures (11°C) and inhibited at high temperatures (16°C). Furthermore, fish held at 16°C failed to exhibit significant production of 17,20βP even in response to luteinizing hormone releasing hormone analogue (LHRHa) treatment. Pankhurst et al. (1996) observed no significant

temperature-related differences in plasma levels of maturational gonadotropin (GtH-II) in trout, supporting the view that elevated temperatures affected steroidogenesis rather than GtH-II synthesis and release. It remains unclear whether the reproductive failure observed in Atlantic salmon is a consequence of temperature effects on pituitary GtH-II secretion, GtH-II-stimulated 20β-HSD activity, or substrate supply to 20β-HSD.

The previous studies on Tasmanian salmon (Chapter 3 and 4) suggested the potential of short-term temperature ramp-down as a means of restoring ovulation in heat-exposed fish. Reduction of holding temperature from 16°C to 8°C was followed by a rapid increase in plasma levels of 17,20βP and ovulation in untreated fish (Chapter 3). Here, although LHRHa-injected fish failed to produce viable ova following temperature reduction (Chapter 4), it is important to note that 35 days had elapsed between LHRHa administration and temperature reduction. Thus it is unlikely that the above observation accurately reflects the likely response of fish if LHRHa treatment and temperature reduction were to commence simultaneously.

With these considerations in mind, the present study was conducted in order to examine more closely the potential of combined temperature ramp-down and LHRHa treatment as a means of ameliorating the effects of exposure to elevated temperature in female Atlantic salmon. LHRHa has been widely applied to advance and/or synchronise ovulation in salmonids (eg Donaldson et al., 1981,1985;. Crim et al., 1983, 1986; Sower et al., 1984; Van Der Kraak et al., 1984,1985; Mylonas et al., 1992) where it acts to stimulate increased GtH-II production. GtH-II, in turn, induces production of 17,20βP via *de novo* synthesis of 20β-HSD. In male Atlantic salmon, consistent with the known role of 17,20βP in the control of milt production, the spermiation response to LHRHa treatment was enhanced by co-administration of 17P as substrate for 17,20βP production (King and Young, 2001). However, since ovulation is probably mediated by a range of factors other than, or in addition to, 17,20βP (reviewed by Goetz and Garczynski, 1997), the effects of co-administration of LHRHa and 17P on ovulation in female salmon are unknown.

In addition to potentially ameliorating thermal insult, co-administration of LHRHa and 17P might distinguish whether thermally impaired fish remain sensitive to LHRHa

and/or possess the capacity to produce 17,20βP. Ovulation or significant modification of 17,20βP production in response LHRHa would indirectly indicate pituitary GtH-II secretion. Similarly, production of 17,20βP in response to 17P would confirm the presence of 20β-HSD activity and, by association, GtH-II. Conversely, failure of 17P treatment to elicit 17,20βP synthesis would tend to confirm the conclusions of Pankhurst et al. (1996) and Pankhurst and Thomas (1998).

Accordingly, pre-ovulatory female Atlantic salmon were held at water temperatures of 11°C or 16°C, and a third group underwent temperature reduction from 16°C to 11°C. Fish were treated first with LHRHa or saline followed by 17P or saline. Blood samples were collected at 48-72h intervals for up to 10 days post first injection and plasma was analysed for levels of the gonadal steroids testosterone (T), 17β-estradiol (E_2) and 17,20βP. Fertility of ova was also assessed following regular ovulation checks.

5.2 Materials & Methods

5.2.i Stock, Husbandry, Temperature and Photoperiod Regimes

One hundred and twenty sexually maturing 2+ female Atlantic salmon (mean weight 5.7kg) were transported from Aquatas Pty. Ltd., (Margate, Tasmania) to SALTAS Freshwater Operations (Wayatinah, Tasmania) in late March 1998 and were maintained in three groups of four temperature-controlled 4m^3 Rathbun tanks (10 fish per tank) initially set at water temperatures of 16°C. Tanks were supplied with partially recirculated water. Biological filtration and water exchanges of 50% per day were employed to prevent the accumulation of toxic metabolites. Oxygen saturation was maintained at 100-120% by the addition of gaseous oxygen via ceramic diffusers. Twenty four hours after transfer to temperature-controlled conditions, the temperature of one system was reduced to 11°C at a rate of 1°C.day^{-1}, then, after a further 20 days, the temperature of a second system was reduced from 16°C to 11°C at a similar rate. Fish in the third group were maintained at 16°C throughout (Figure 5.1). Fish were exposed to a simulated natural photoperiod (42° S) for the duration of the study.

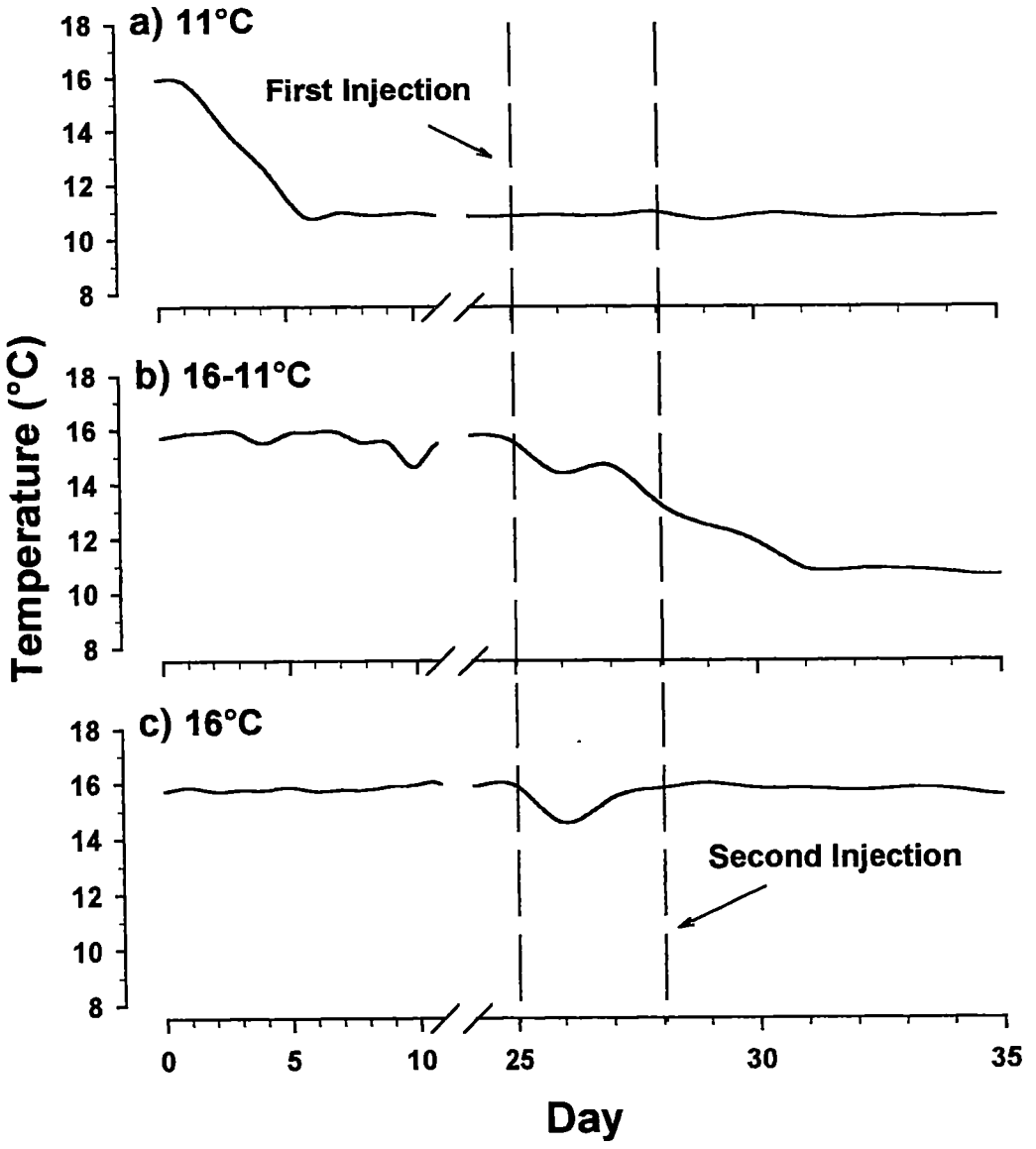

Figure 5.1 Water temperatures experienced by female Atlantic salmon maintained at **a**) 11°C, **b**) exposed to temperature reduction from 16 to 11°C after 25 days, or **c**) maintained at 16°C throughout.

5.2.ii Treatments

Twenty-five days after transfer to the temperature-controlled systems, all fish from each of the three temperatures were anaesthetised (25p.p.m. benzocaine), weighed (± 0.1kg) and tagged by placing visible implant tags (VI Tags, Northwest Marine Technology Inc, Shaw Island, WA) in the left adipose eyelid according to the methods

described by Bergman et al. (1992) and Kincaid and Calkins (1992). Blood samples were also taken from each fish by puncture of the duct of Cuvier (Lied et al., 1975) using heparinized (lithium heparin) syringes and 22G needles. After centrifugation, the resulting plasma was stored at -20°C prior to analysis of steroid levels. In addition, fish from each temperature regime were injected i.p. with either saline (n = 20) or with a solution of des-Gly^{10}[D-Ala^{6}]-luteinizing hormone releasing hormone ethylamide (LHRHa, Sigma) in saline (n = 20). Three days later, anaesthesia and blood sampling were repeated and fish were injected with saline or with a saline suspension of 17α-hydroxyprogesterone (17P) such that the following four treatment groups (n = 10) were created; Saline + Saline, Saline + 17P, LHRHa + Saline, LHRHa + 17P. As fish were individually marked hormone treatments were mixed within holding tanks in order to eliminate tank effects. Injection volume was 500μl.kg^{-1} body weight, and the dose for the LHRHa and 17P treatments was 25 μg.kg^{-1} and 1.0 mg.kg^{-1} body weight respectively. Anaesthesia and blood sampling were repeated at 48-72 hour intervals until 10 days post first injection. Ovulation checks were conducted at similar intervals and continued until 20 days post first injection.

5.2.iii Ova Fertilisation

Fish that expressed ova in response to the gentle application of pressure to the abdomen were transferred to a holding tank maintained at 8°C. After 24h at 8°C, fish were killed by a blow to the head, towelled dry and ova were expressed into a stainless steel sieve. In order to facilitate ova collection, a 2cm cut was made at the genital papilla. Ova were transferred to a stainless steel bowl and fertilised using pooled milt from 3-4 naturally spermiating males. Ova and milt were gently mixed and water (500ml) was added to ensure sperm activation. After 2 mins, ova were rinsed with clean water then left to water-harden for 60 mins. After water-hardening, sample batches (100-200) of ova from each female were incubated at 8°C for 120 degree-hours and fertilisation (%) was determined on the basis of first cell division, visualised by treatment with a clearing solution (1:1:1 v/v methanol:acetic acid:water) for 2 mins.

5.2.iv Plasma Steroid Measurement

Plasma levels of E_2, T and 17,20βP were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Carragher (1992). Extraction efficiency (mean recovery of ^{3}H-labelled steroid from triplicates of a plasma pool) was 73-87, 89-96 and 89-99% for E_2, T and 17,20βP respectively and values for each steroid were adjusted accordingly. Interassay variability measured using aliquots of a pooled internal standard was (%CV(n)) 5.9(9), 11.6(9) and 12.4(9) for E_2, T and 17,20βP respectively.

5.2.v Statistical Analysis

Where appropriate, data were analysed by one-way and two-way ANOVA and Tukey's HSD tests using the SYSTAT 8.0 for Windows computer package. However, due to the requirement by repeated measures ANOVA for a full orthogonal design with balanced sample numbers (Potvin et al., 1990), mortality and removal of ovulated fish from the experiment precluded analysis of hormone data using this method. Similarly, the removal of ovulated fish hindered the application of two-way ANOVA to hormone data. Therefore, the occurrence of treatment-related differences in plasma hormone levels over the course of the study was evaluated using an analysis of residual sums of squares (ARSS) according to the methods of Chen et al. (1992). The procedure for the comparison of pairs of treatments using the ARSS was as follows: (1) a second-order polynomial function was fitted to the data for each treatment, (2) residual sum of squares (RSS) and associated degrees of freedom (DF) were calculated for each treatment then summed to yield the total RSS and DF, (3) data for both treatments were pooled to calculate the RSS and DF of a single polynomial function fitted to the combined data, and (4) an *F*-statistic was calculated as

$$F = \frac{\dfrac{RSS_p - RSS_s}{DF_{RSS_p} - DF_{RSS_s}}}{\dfrac{RSS_s}{DF_{RSS_s}}} = \frac{\dfrac{RSS_p - RSS_s}{3(K-1)}}{\dfrac{RSS_s}{N-3K}}$$

where RSS_p = RSS of the polynomial function fitted to pooled data, RSS_s = sum of the separate RSS of the functions fitted to the data from each treatment, K = the

number of treatments in the comparison (ie. 2), and N = total sample size. The calculated F value was then compared with the critical F, with DFs of 3(K - 1) and N - 3K.

In spite of the assertion by Potvin et al. (1990) that such a curve-fitting approach to the analysis of repeated measures data is distribution free, the robustness of the theoretical F-distribution in the above analysis was confirmed using a randomization procedure. Sample data were repeatedly randomized (a total of 5,000 iterations were conducted) and a series of new F values were calculated as described above. The new F values were then used to generate an empirical frequency distribution of the sampling statistic which was in turn, compared to the theoretical distribution in order to assess the possible extent of any deviations arising from the correlation existing among the data. As a further safeguard against Type I error, pairwise comparisons were only made between temperature regimes within hormone treatment regimes, and between hormone treatment regimes within temperature regimes such that only 30 of a possible 54 pairwise comparisons were made. Finally, proportion data were normalised by arcsin transformation.

5.3 Results

5.3.i Ovulation

In fish maintained at 11°C throughout, ovulations commenced first in fish which received injections of LHRHa and 17,P where the first ovulation occurred 5 days after first injection and all fish ovulated within 2 further days (Figure 5.2a). In fish injected with LHRHa and saline, ovulations also commenced 5 days after first injection, but were not completed until 10 days after first injection. In fish treated with saline plus 17P, ovulations commenced 7 days post first injection and this group completed ovulation on day 12. In contrast, saline-injected controls commenced ovulation on day 5 but did not complete ovulation until 17 days after first injection. In fish maintained first at 16°C, then reduced to 11°C, the first ovulations occurred 5 days after first injection in fish which received LHRHa plus saline (Figure 5.2b), and ovulations were complete 10 days after first injection. The group which received LHRHa and 17P ovulated between 7 and 14 days after first injection. In fish treated with saline followed by 17P, ovulations commenced 10 days post first injection and were

completed on day 14. As observed at 11°C, sham-injected controls were the last fish to ovulate with ovulations commencing on day 12 and not reaching completion until day 19 after first injection. There were no ovulations in any treatment group among fish maintained at 16°C throughout.

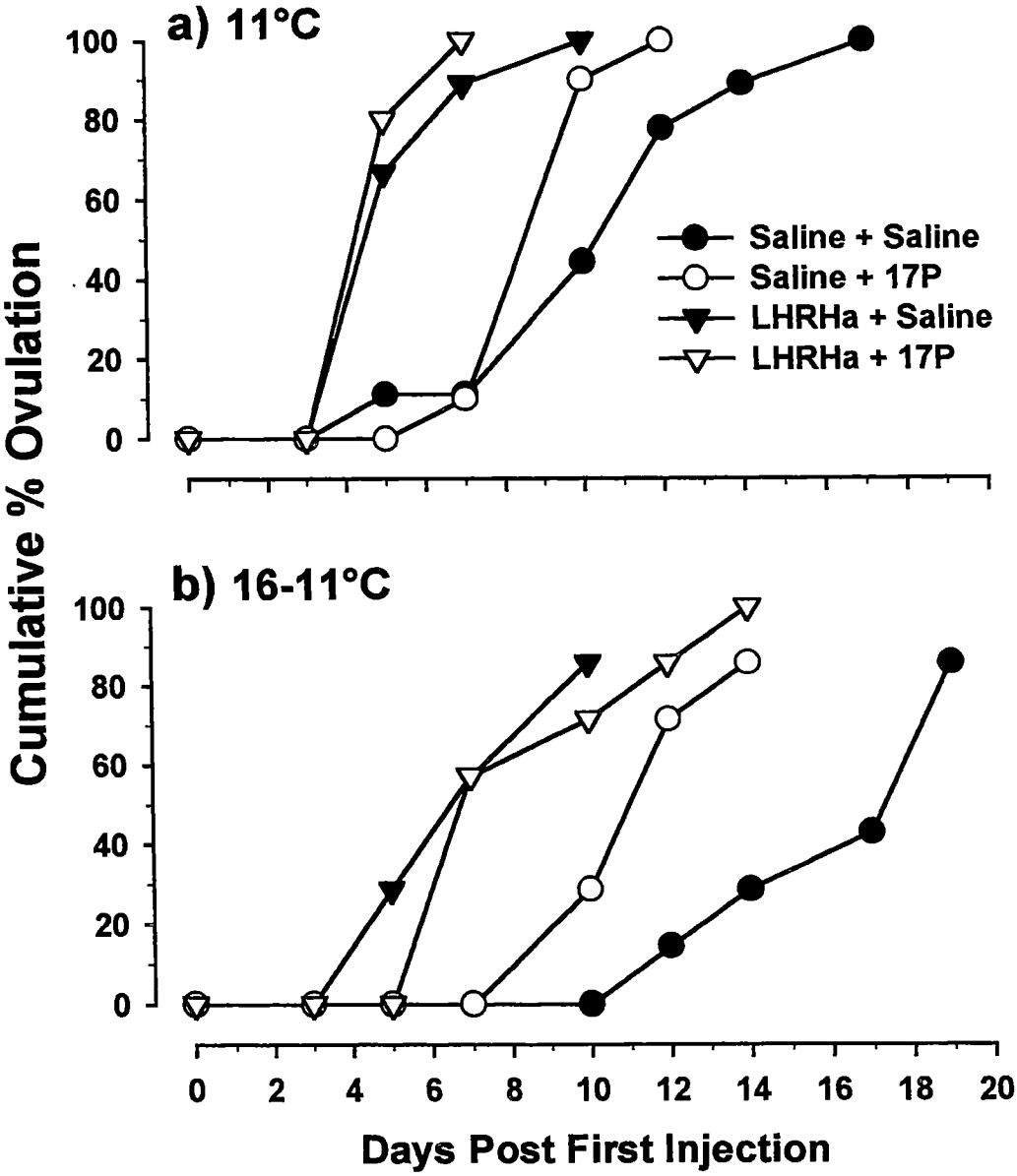

Figure 5.2 Cumulative percent ovulation in female Atlantic salmon maintained at **a)** 11°C or **b)** exposed to temperature reduction from 16 to 11°C, and treated with saline only (●), saline and 17P (○), LHRHa and saline (▼), or LHRHa and 17P (▽).

5.3.ii Ova Fertility

The mean fertilities of ova from fish maintained at 11°C were consistently high (> 80%) and there were no significant differences between treatments (Figure 5.3a; Table 5.1). In contrast, treatment with saline only was associated with a marked reduction in mean egg fertility in fish which underwent temperature reduction (Figure 5.3b; Table 5.1). Under temperature reduction, treatment with 17P alone tended to result in a partial restoration of fertility (Figure 5.3b), while treatment with LHRHa, alone or in combination with 17P, resulted in mean egg fertilities which were significantly higher than those of controls and not significantly different to those obtained at 11°C (Figure 5.3a and b).

Table 5.1 ANOVA table for Two-Way ANOVA of percent egg fertility for female Atlantic salmon maintained at 11°C or exposed to temperature reduction from 16°C to 11°C and treated with LHRHa and/or 17P.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio	P
Temperature Regime	4315.7	1	4315 7	13.220	0.001
Hormone Treatment	4021.2	3	1340.4	4.106	0.011
Temperature Regime x Hormone Treatment	2215.1	3	738.4	2.262	0.092
Error	16976.0	52	326.5		

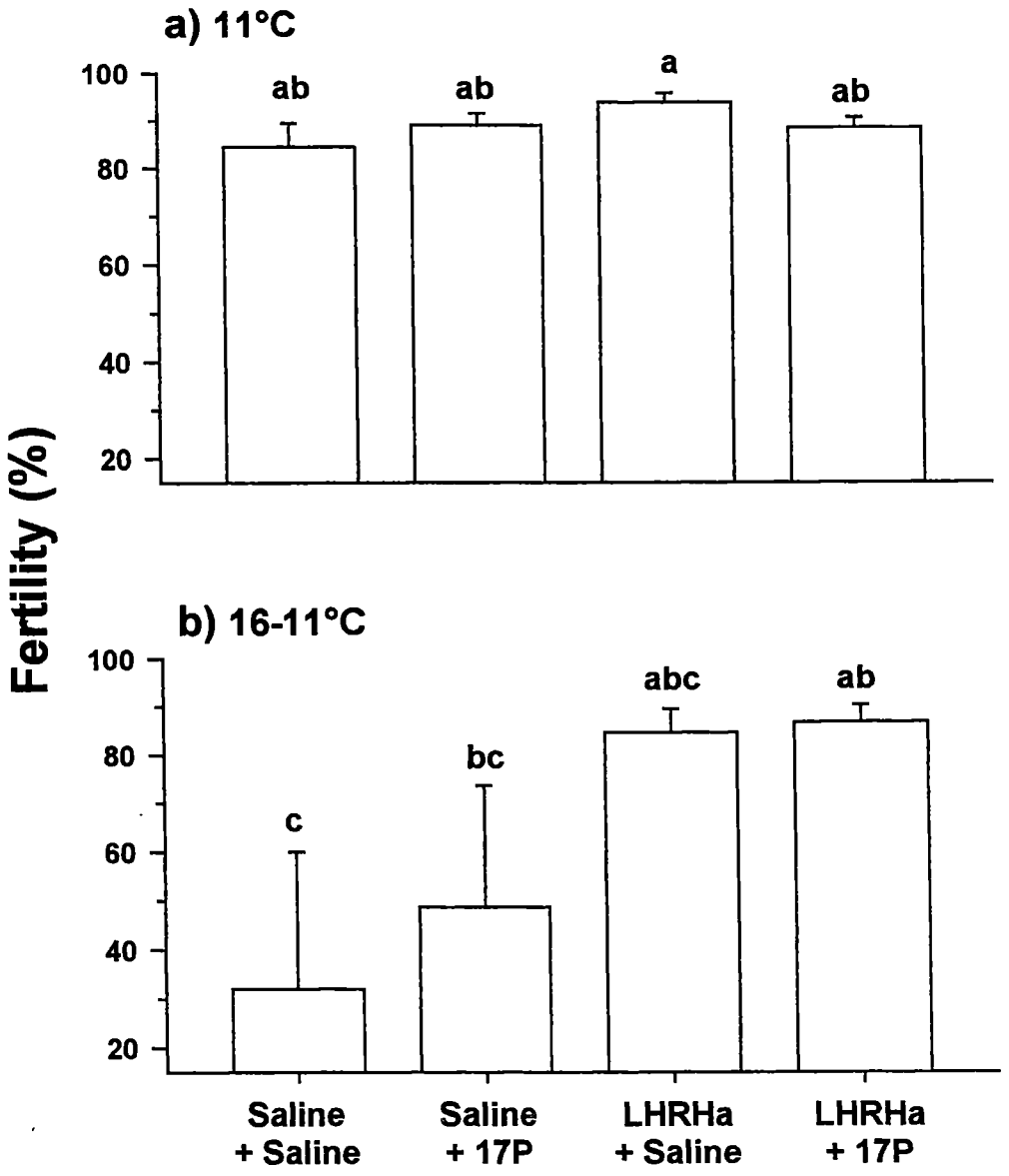

Figure 5.3 Mean (+ S.E.M.) percent fertility of ova from female Atlantic salmon maintained at **a)** 11°C or **b)** exposed to temperature reduction from 16 to 11°C following treatment with saline only, saline and 17P, LHRHa and saline or LHRHa and 17P. N = 6-10, bars with the same superscript are not significantly different (P>0.05).

5.3.iii Plasma Steroid Levels

At all temperatures and in all hormone treatments plasma E_2 tended to decline from levels of approximately 10-20 $ng.ml^{-1}$ at the commencement of sampling to below 5 $ng.ml^{-1}$ at the completion of sampling (Figure 5.4). In saline-injected controls (Figure 5.4a), maintenance at 16°C was associated with E_2 levels which declined more rapidly than those in fish held at 11°C or in fish exposed to temperature reduction ($P = 0.0067$ and 0.0223 respectively, Table 5.2). However, this difference was largely due to a relatively low starting E_2 level at 16°C (~ 11 $ng.ml^{-1}$ cf. 14-15 $ng.ml^{-1}$). Similarly, in fish which received saline in combination with 17P (Figure 5.4b), E_2 production at 16°C also declined more rapidly than that observed in fish which underwent temperature reduction ($P = 0.0117$, Table 5.2). Here however, the difference appeared to be due to a relatively elevated starting E_2 level at 16°C (~ 19 $ng.ml^{-1}$ cf. 13-16 $ng.ml^{-1}$). Treatment with LHRHa, either alone (Figure 5.4c) or in combination with 17P (Figure 5.4d) resulted in no significant temperature-related differences in plasma E_2 production (Table 5.2).

Comparison of hormone treatment regimes within temperature regimes (Table 5.3) indicated that at 11°C, LHRHa, when administered in combination with saline, resulted in a more rapid fall in E_2 than occurred saline-injected controls ($P = 0.0056$, Table 5.3), while, relative to 17P-treated animals, the increase in decline approached significance ($P = 0.0545$, Table 5.3). Under combined LHRHa/17P treatment, E_2 production fell more rapidly than that in both saline-injected controls and 17P-treated animals ($P = 0.0001$ and 0.0005 respectively, Table 5.3). Similarly, under temperature reduction, both LHRHa treatments were associated with a significantly faster decline in E_2 production relative to saline-injected controls ($P = 0.0138$ and 0.0082, Table 5.3). Here however, only combined LHRHa/saline treatment resulted a reduction in E_2 production which was more rapid than that of 17P-treated stock ($P = 0.0292$, Table 5.3). At 16°C, due largely to a high starting E_2 level, treatment with saline in combination with 17P resulted in a more rapid decrease in E_2 than in controls ($P < 0.0001$, Table 5.3) or both LHRHa treatments ($P = 0.0084$ and 0.0049, Table 5.3).

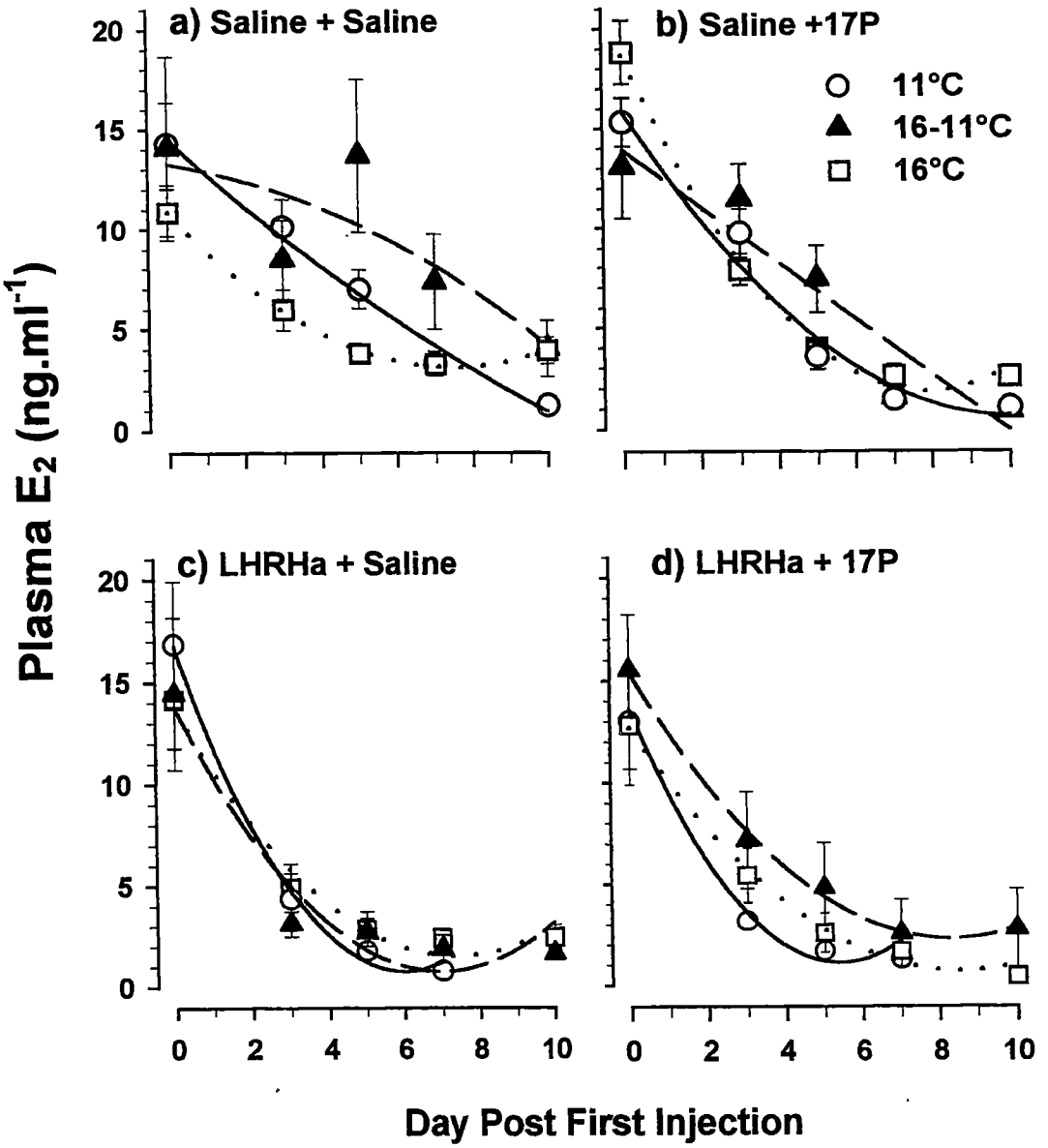

Figure 5.4 Mean (± S.E.M.) plasma E_2 (ng.ml^{-1}) in female Atlantic salmon maintained at 11°C (○), exposed to temperature reduction from 16 to 11°C (▲), or held at 16°C (□) and treated with **a)** saline only, **b)** saline and 17P, **c)** LHRHa and saline, or **d)** LHRHa and 17P. Lines represent best fit 2nd order polynomial functions at 11°C (——), temperature reduction (- -) and 16°C (· ·).

Table 5.2 Matrix of probabilities for pairwise within-treatment regime comparisons of plasma levels of E_2, T and 17,20βP in female Atlantic salmon held at 11 or 16°C or exposed to temperature ramp-down from 16 to 11°C. Associated F-values and degrees of freedom are displayed in parentheses.

Steroid		Treatment Regime							
		Saline + Saline		Saline + 17P		LHRHa + Saline		LRHa + 17P	
	Temperature (°C)	16-11	16	16-11	16	16-11	16	16-11	16
E_2	**11**	**0.2161** ($F_{3,72}$= 1.52)	**0.0007** ($F_{3,72}$= 6.31)	**0.1542** ($F_{3,78}$= 1.80)	**0.0874** ($F_{3,77}$= 2.27)	**0.7936** ($F_{3,50}$= 0.34)	**0.6378** ($F_{3,52}$= 0.57)	**0.1733** ($F_{3,54}$= 1.72)	**0.6784** ($F_{3,52}$= 0.51)
	16-11	-	**0.0223** ($F_{3,64}$= 3.42)	-	**0.0117** ($F_{3,63}$= 3.97)	-	**0.9474** ($F_{3,48}$= 0.12)	-	**0.5974** ($F_{3,48}$= 0.63)
T	**11**	**0.1038** ($F_{3,72}$= 2.13)	**0.1643** ($F_{3,72}$= 1.75)	**0.0008** ($F_{3,78}$= 6.19)	**0.0202** ($F_{3,77}$= 3.47)	**0.0498** ($F_{3,50}$= 2.79)	**0.2558** ($F_{3,52}$= 1.39)	**<0.0001** ($F_{3,54}$= 9.61)	**0.0034** ($F_{3,52}$= 5.17)
	16-11	-	**0.0050** ($F_{3,64}$= 4.69)	-	**0.0216** ($F_{3,63}$= 3.46)	-	**0.5652** ($F_{3,48}$= 0.69)	-	**0.5093** ($F_{3,48}$= 0.78)
17,20βP	**11**	**0.1769** ($F_{3,72}$= 1.69)	**0.0010** ($F_{3,72}$= 6.00)	**0.5349** ($F_{3,78}$= 0 73)	**0.0010** ($F_{3,77}$= 6.00)	**0.0067** ($F_{3,50}$= 4.56)	**<0.0001** ($F_{3,52}$= 13.46)	**<0.0001** ($F_{3,54}$= 16.13)	**<0.0001** ($F_{3,52}$= 18.85)
	16-11	-	**0.0013** ($F_{3,64}$= 5.88)	-	**0.0154** ($F_{3,63}$= 3.74)	-	**0.1017** ($F_{3,48}$= 2.19)	-	**0.1069** ($F_{3,48}$= 2.14)

Plasma levels of T also varied with temperature and treatment regime (Figure 5.5). In controls (Figure 5.5a), plasma T tended to decline in fish held at 16°C whereas, in fish which underwent temperature reduction, T levels tended to remain above ~35 $ng.ml^{-1}$. Similarly, in fish held at 11°C plasma T also tended to remain elevated and only declined on the last day of sampling. As a result, the pattern of plasma T production observed at 16°C differed significantly from that observed under temperature reduction ($P = 0.0050$, Table 5.2). In 17P-treated animals (Figure 5.5b), plasma T declined to levels below ~ 20 $ng.ml^{-1}$ at all temperatures, but the patterns of decline were significantly different at all temperatures ($P < 0.025$, Table 5.2). Plasma T levels fell first in fish held at 16°C, followed by those in fish held at 11°C whereas, under temperature reduction plasma tended not to fall until after 6 days post first injection (Figure 5.5b). Plasma T levels also declined in fish which received LHRHa in combination with saline (Figure 5.5c). In particular, in fish maintained at 11°C, plasma T declined more rapidly than in fish which underwent temperature reduction ($P = 0.0498$, Table 5.2). However, there was no significant difference relative to fish held at 16°C. In fish which received combined LHRHa/17P treatment (Figure 5.5d), plasma T also declined as sampling progressed. Here the decline observed in fish held at 11°C was more rapid than that both in fish which underwent temperature reduction ($P < 0.0001$, Table 5.2) and in fish maintained at 16°C ($P = 0.0034$, Table 5.2).There were also significant treatment-related differences in plasma T levels among fish held at 11°C (Table 5.3), with plasma T levels not different in those treatments which incorporated LHRHa ,whereas all other pairs of treatments differed significantly ($P \leq 0.0002$, Table 5.3). As a result, the decline in plasma T in both groups of LHRHa-treated fish preceded that in 17-P treated fish which in turn, preceded that in controls. In contrast, in fish which underwent temperature reduction there were no significant differences between hormone treatments although the decline in plasma T in all three hormone treatments preceded that in saline-injected controls ($P \leq 0.0074$, Table 5.3). Similarly, there were no significant differences between any of the hormone treatments at 16°C and only combined LHRHa/saline treatment differed significantly from controls ($P = 0.0329$, Table 5.3) while the combined LHRHa/17P treatment approached significance relative to controls ($P = 0.0507$, Table 5.3).

Table 5.3 Matrix of probabilities for pairwise within-temperature regime comparisons of plasma levels of E_2, T and 17,20βP in female Atlantic salmon treated with LHRHa and/or 17P. Associated F-values and degrees of freedom are displayed in parentheses.

Steroid	Treatment	Temperature Regime: 11°C			16-11°C			16°C		
		Saline + 17P	LHRHa + Saline	LHRHa + 17P	Saline + 17P	LHRHa + Saline	LHRHa + 17P	Saline + 17P	LHRHa + Saline	LHRHa + 17P
E2	**Saline + Saline**	**0.1894** ($F_{3,69}$=1.63)	**0.0056** ($F_{3,59}$=4.63)	**0.0001** ($F_{3,61}$=8.22)	**0.2650** ($F_{3,64}$=1.35)	**0.0138** ($F_{3,57}$=3.86)	**0.0082** ($F_{3,60}$=4.30)	**<0.0001** ($F_{3,64}$=16.43)	**0.1386** ($F_{3,61}$=1.90)	**0.2636** ($F_{3,57}$=1.36)
	Saline + 17P	-	**0.0545** ($F_{3,64}$=2.68)	**0.0005** ($F_{3,66}$=6.80)	-	**0.0292** ($F_{3,57}$=3.22)	**0.4418** ($F_{3,60}$=0.91)	-	**0.0084** ($F_{3,61}$=4.27)	**0.0049** ($F_{3,57}$=4.77)
	LHRHa + Saline	-	-	**0.4743** ($F_{3,56}$=0.85)	-	-	**0.5245** ($F_{3,58}$=0.75)	-	-	**0.8796** ($F_{3,54}$=0.22)
T	**Saline + Saline**	**0.0002** ($F_{3,69}$=7.37)	**<0.0001** ($F_{3,59}$=12.22)	**<0.0001** ($F_{3,61}$=18.33)	**0.0017** ($F_{3,64}$=5.65)	**0.0058** ($F_{3,57}$=4.63)	**0.0074** ($F_{3,60}$=4.39)	**0.0970** ($F_{3,64}$=2.20)	**0.0329** ($F_{3,61}$=3.11)	**0.0507** ($F_{3,57}$=2.75)
	Saline + 17P	-	**0.0001** ($F_{3,64}$=8.19)	**<0.0001** ($F_{3,66}$= 17.11)	-	**0.2901** ($F_{3,57}$=1.28)	**0.7730** ($F_{3,60}$=0.37)	-	**0.8207** ($F_{3,61}$=0.31)	**0.6466** ($F_{3,57}$=0.56)
	LHRHa + Saline	-	-	**0.3965** ($F_{3,56}$=1.01)	-	-	**0.8443** ($F_{3,58}$=0.27)	-	-	**0.4959** ($F_{3,54}$=0.81)
17,20βP	**Saline + Saline**	**<0.0001** ($F_{3,69}$=19.03)	**<0.0001** ($F_{3,59}$=15.85)	**<0.0001** ($F_{3,61}$=36.21)	**<0.0001** ($F_{3,64}$=22.40)	**<0.0001** ($F_{3,57}$=10.17)	**<0.0001** ($F_{3,60}$=32.87)	**<0.0001** ($F_{3,64}$=14.57)	**0.0004** ($F_{3,61}$=6.95)	**<0.0001** ($F_{3,57}$=36.89)
	Saline + 17P	-	**0.0002** ($F_{3,64}$=7.85)	**<0.0001** ($F_{3,66}$=20.94)	-	**0.2039** ($F_{3,57}$=1 58)	**0.7704** ($F_{3,60}$=0.38)	-	**0.0244** ($F_{3,61}$=3 36)	**0.2610** ($F_{3,57}$=1.37)
	LHRHa + Saline	-	-	**0.0753** ($F_{3,56}$=2.42)	-	-	**0.0379** ($F_{3,58}$=3.02)	-	-	**<0.0001** ($F_{3,54}$=9.34)

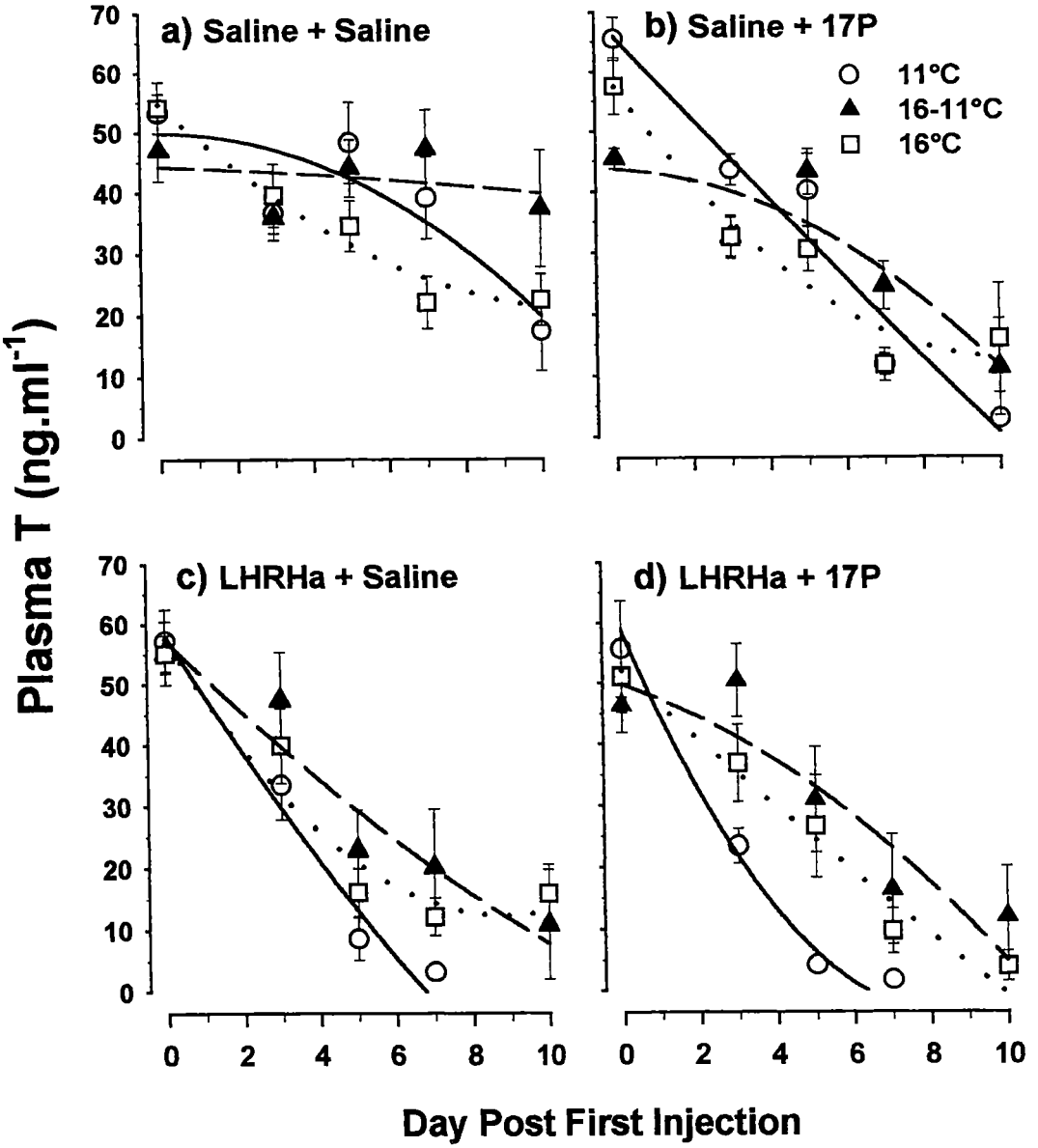

Figure 5.5 Mean (± S.E.M.) plasma T ($ng.ml^{-1}$) in female Atlantic salmon maintained at 11°C (○), exposed to temperature reduction from 16 to 11°C (▲), or held at 16°C (□) and treated with **a)** saline only, **b)** saline and 17P, **c)** LHRHa and saline, or **d)** LHRHa and 17P. Lines represent best fit 2nd order polynomial functions at 11°C (——), temperature reduction (- -) and 16°C (· ·).

At all temperatures and in all treatments, plasma 17,20βP levels were initially near or below assay detection limits (~ 0.3 $ng.ml^{-1}$, Figure 5.6). In saline-treated controls (Figure 5.6a), moderate levels of 17,20βP (up to ~ 30 $ng.ml^{-1}$) were observed towards the end of sampling in fish held at 11°C and in fish which underwent temperature reduction, whereas no increase in 17,20βP was observed at 16°C. As a result, 17,20βP production in fish held at 11°C and in fish which underwent temperature reduction differed significantly ($P = 0.0010$ and 0.0013 respectively, Table 5.2) from that at 16°C. Similarly, in fish which received saline and 17P (Figure 5.6b), 17,20βP production in fish held at 11°C (up to ~ 105 $ng.ml^{-1}$) and in fish which underwent temperature reduction (up to ~ 85 $ng.ml^{-1}$) significantly exceeded ($P = 0.0010$ and 0.0154 respectively, Table 5.2) that observed in fish held at 16°C, in spite of measurable 17,20βP production (up to ~ 45 $ng.ml^{-1}$) at 16°C. In contrast, in fish which received LHRHa along with saline (figure 5.6c), there was no significant difference in 17,20βP production between fish which underwent temperature reduction and those maintained at 16°C throughout. However, 17,20βP production in fish held at 11°C (up to ~ 70 $ng.ml^{-1}$) was significantly higher than that observed under either of the other temperature regimes ($P \leq 0.0067$, Table 5.2). A similar but even more significant ($P < 0.0001$, Table 5.2) pattern of 17,20βP production was observed in fish which received the combined LHRHa/17P treatment (up to ~ 90 $ng.ml^{-1}$, Figure 5.6d). As previously observed for T, 17,20βP production at 11°C was not different in those treatments which incorporated LHRHa (although the comparison approached significance; $P = 0.0753$, Table 5.3) whereas all other pairs of treatments differed significantly ($P \leq 0.0002$, Table 5.3). Under temperature reduction, 17,20βP production in response to 17P treatment was not different from that observed in both LHRHa treatments whereas, the LHRHa treatments differed significantly ($P = 0.0379$, Table 5.3) and all three hormone treatments resulted in patterns of 17,20βP production which differed from that of saline-injected controls in a manner which was highly significant ($P < 0.0001$, Table 5.3). Similar treatment-related differences in plasma 17,20βP levels occurred at 16°C where only those treatments incorporating 17P resulted in similar 17,20βP production (Table 5.3).

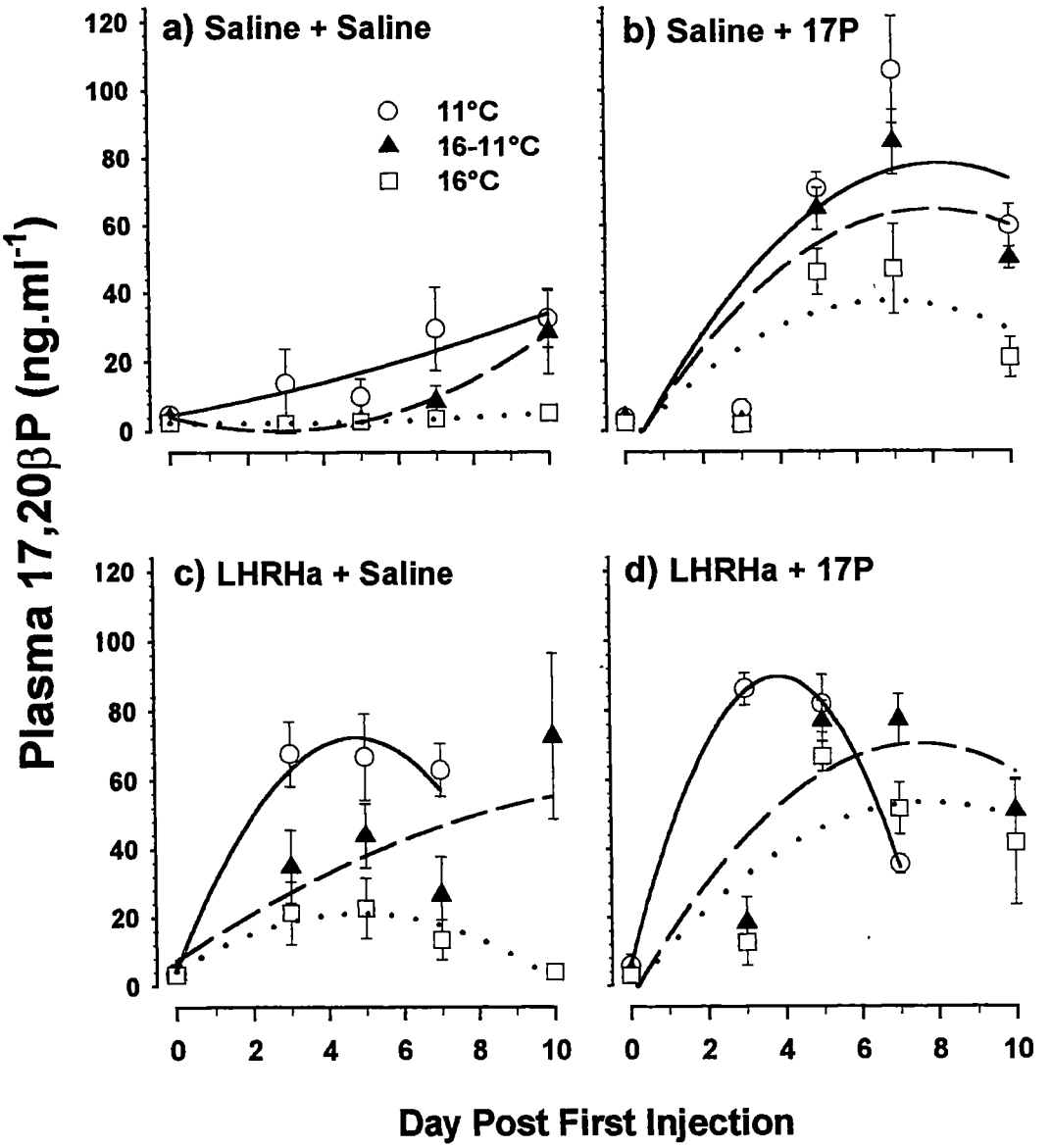

Figure 5.6 Mean (± S.E.M.) plasma 17,20βP ($ng.ml^{-1}$) in female Atlantic salmon maintained at 11°C (○), exposed to temperature reduction from 16 to 11°C (▲), or held at 16°C (□) and treated with **a)** saline only, **b)** saline and 17P, **c)** LHRHa and saline, or **d)** LHRHa and 17P. Lines represent best fit 2nd order polynomial functions at 11°C (——), temperature reduction (- -) and 16°C (· ·).

5.3.iv Fish Survival

Survival to ovulation was 80% or greater in fish maintained at 11°C and in those which underwent temperature reduction from 16°C to11°C, irrespective of hormone treatment. In contrast, survival to the end of the study in fish held at16°C throughout tended to differ between hormone treatments. 60% of sham-injected controls survived to the completion of the study whereas only 30% of fish treated with LHRHa alone survived to the cessation of ovulation checks. Furthermore, only one fish (10%) from each of the treatments where 17P was administered survived to the end of the study.

5.4 Discussion

Short-term temperature reduction restored ovulation in female Atlantic salmon previously maintained at 16°C to the extent that their pattern of cumulative percentage ovulation closely resembled that observed in fish held at 11°C throughout. Under both the 11°C, and temperature reduction regimes, saline-treated controls ovulated within 19 days of first injection while treatment with 17P and LHRHa, alone or in combination, resulted in progressive advances in ovulation. These results are consistent with the previously observed restoration of ovulation in female Atlantic salmon following temperature reduction from 16°C to 8°C (Chapters 3 and 4). Similarly, the ovulation of LHRHa-treated fish under both temperature regimes agrees with the widely recognised effectiveness of LHRHa treatment for inducing ovulation in salmonids (eg Donaldson et al., 1981,1985;. Crim et al., 1983, 1986; Sower et al., 1984; Van Der Kraak et al., 1984,1985; Mylonas et al., 1992). In particular, Taranger et al. (1992) observed that ovulation in Atlantic salmon maintained at natural temperatures was advanced by up to 14 days following LHRHa treatment with the greatest synchronising effect of LHRHa administration occurring when treatments were applied close to the time of ovulation in controls.

In vivo application of 17P, alone or in combination with LHRHa, has not been previously reported in female salmonids and the observed advancement of ovulation in response to the application of 17P is somewhat unexpected since there is no evidence that 17,20βP directly stimulates ovulation in salmonids. 17,20βP can stimulate

ovulation in the yellow perch (*Perca flavescens*) (Goetz, 1983) and the goldeye (*Hiodon alosoides*) (Pankhurst, 1985) but in salmonids, ovulation is probably mediated by other acknowledged regulators of oocyte expulsion such as eicosanoids (in particular prostaglandin (PG) $F_{2\alpha}$) which are synthesised at the time of ovulation in response to hormonal (GtH-II) stimulation (reviewed by Goetz and Garczynski, 1997). Nevertheless, it is likely that the advancement of ovulation observed in response to the application of 17P in the present study results from its conversion to 17,20βP and the subsequent effects on oocyte maturation. In this regard, in male Atlantic salmon, plasma levels of 17,20βP were increased 2 to 10-fold in 17P-treated fish (King and Young, 2001) while in snapper, plasma 17,20βP levels in 17P-treated fish increased almost 40-fold within two hours of injection (Pankhurst, 1994). During the present study, plasma 17,20βP levels in 17P-treated fish consistently exceeded those of control or LHRHa-treated animals under each temperature regime, indicating conversion of 17P to 17,20βP.

A second possibility is that 17P also had a direct effect on maturation. 17P has been demonstrated to possess limited binding affinity for teleost maturation-inducing steroid (MIS) receptors and to exhibit limited maturation inducing activity. For example, Canario and Scott (1988) reported that the potency of 17P in inducing *in vitro* maturation of rainbow trout oocytes was 2-5% of that of 17,20βP. Thomas (2000) presented data from the spotted seatrout (*Cynoscion nebulosus*) showing that the affinity of 17P for the nuclear MIS receptor was ~ 10% of that of 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), the MIS in that species. Plasma levels of 17P were not assessed during the present study. However, since the quantity of 17P used might be regarded to represent a pharmacological dose, plasma 17P levels may have been sufficiently elevated to result in a direct maturational effect.

The consistently high fertility of ova from fish held at 11°C, irrespective of hormone treatment, is in agreement with previous results (Chapters 3 and 4) as well as those for Arctic charr (*Salvelinus alpinus)* reported by Gillet (1991) and those for Atlantic salmon reported by Taranger and Hansen (1993) and Taranger et al. (2000). During previous studies on Tasmanian salmon (Chapters 3 and 4) the fertility of ova from fish held at or below 11°C exceeded 80%, while in charr, maintenance at, or early transfer

to, 5°C resulted in high egg survival (> 75%) relative to that associated with later transfer to 5°C (< 70%) or maintenance at 8°C throughout the periovulatory period (< 60%; Gillet, 1991). Taranger and Hansen (1993) reported increased egg survival associated with the transfer of female Atlantic salmon to below-natural temperatures (> 90%) while, in turn, natural temperatures were associated with higher survivals (~ 85%) than elevated temperatures (< 80%). Similarly, in a study of the combined effects of accelerated photoperiod and reduced temperature on spawning in female Atlantic salmon, Taranger et al. (2000) observed a significant reduction in the survival of ova from fish which had experienced advanced photoperiod at natural temperature (34.5%) relative to those which had been held at ~5°C below natural temperature (68.9%).

The present observation of reduced fertility in ova from saline-treated fish which underwent temperature ramp-down is also consistent with the reported effect of delayed temperature reduction on egg survival in the Arctic charr (Gillet, 1991) discussed above. However, the restoration of egg viability following LHRHa treatment contrasts with previous results (Chapter 4) where the fertility of sham-treated controls exceeded that of ova from LHRHa-injected animals. Importantly, the previous data were obtained from only 2 fish which underwent temperature reduction more than 35 days after LHRHa injection, whereas the present study involved simultaneous LHRHa administration and temperature reduction. Furthermore, there was also a tendency towards partial restoration of egg viability associated with 17P treatment in fish which underwent temperature reduction. Thus, a GtH-II- and/or steroid-mediated mechanism appears to have operated to enhance egg viability. In this regard, in salmonids, post-ovulatory levels of GtH-II can exceed pre-ovulatory levels (eg. Goetz et al., 1987; Liley and Rouger, 1990) and post-ovulatory levels of GtH-II of spent females can be lower than those of females which retain ova (reviewed by Goetz, 1983). Accordingly, the post-ovulatory surge in GtH-II levels may play a role in the maintenance of the viability of oocytes prior to their release during spawning (reviewed by Hobby and Pankhurst, 1997).

Consistent with previous observations in Atlantic salmon (Chapters 3 and 4) and observations in rainbow trout reported by Pankhurst et al. (1996) and Pankhurst and

Thomas (1998), no ovulations occurred in fish held at 16°C throughout, irrespective of hormone treatment.

The effect of holding temperature on the endocrine response to hormone treatment is best interpreted against the changes seen in saline-injected controls. In general, the patterns of plasma steroid production observed in fish held at 11°C and in fish exposed to temperature reduction agreed with the general pattern of salmonid periovulatory steroid production. Here, E_2 levels are expected to decline while T levels display a transitory peak associated with the fall in aromatization to E_2, followed by a decline (Fostier et al., 1983). Simultaneously, plasma levels of the progestin 17,20βP increase sharply to high levels under GtH-II stimulation (reviewed by Swanson, 1991). In fish in the present study held at 11°C, plasma levels of E_2 and T declined rapidly following the commencement of sampling, and an increase in plasma 17,20βP levels occurred within 36 h. Under temperature reduction, plasma levels of E_2 and T also declined but at a slower rate than at 11°C. Plasma levels of 17,20βP were also slower to increase. Thus, fish held at 11°C throughout, appear to have been well-progressed towards maturation and to have made the GtH-II-stimulated shift in ovarian steroidogenesis away from the 17α-hydroxylase/17-20 lyase cytochrome P-450 ($P450_{C17}$), 17β-hydroxysteroid-dehydrogenase (17β-HSD), and aromatase cytochrome P450 ($P450_{arom}$) enzyme systems which catalyse the conversion of 17P to E_2, to 20β-HSD which catalyses the conversion of 17P to 17,20βP (reviewed by Nagahama, 1997). Similarly, the switch in ovarian steroidogenesis occurred in fish which underwent temperature reduction, but apparently not until ~7 days after the commencement of sampling. Meanwhile, as might be expected, treatment with LHRHa and/or 17P accelerated these processes under both the 11°C and temperature reduction regimes.

In saline-injected fish held at 16°C throughout, plasma levels of E_2 and T also declined rapidly in a manner similar to that observed at 11°C. However, consistent with previous studies (Chapter 3 and 4; Pankhurst and Thomas, 1988) there was no concomitant production of 17,20βP. As a result, it might be concluded that fish held at 16°C lacked adequate GtH-II and failed to make the steroidogenic shift to 20β-HSD. Thus the declining E_2 and T levels in these fish may have simply reflected a

temperature- or handling-related depression of general ovarian steroidogenic capacity. In this context, however, it is noteworthy that treatment of fish held at 16°C with 17P alone resulted in mean plasma levels of 17,20βP which peaked above 45 $ng.ml^{-1}$ within 48 hours. Since 17,20βP production is dependent on GtH-II-stimulated *de novo* synthesis of 20β-HSD (reviewed by Nagahama, 1997), the increase in 17,20βP in animals which received 17P only, indicates some elevation of GtH-II levels in fish maintained at 16°C. Importantly, the relatively low levels of 17,20βP observed in fish which received LHRHa only and the increase in 17,20βP observed when LHRHa and 17P treatment were combined, suggest that 17,20βP production may have been substrate-limited and that there may have been a blockade in the pathway of steroid synthesis above the level of 17P production. This is supported by the fact that LHRHa alone did not augment 17,20βP production to the same extent as 17P treatment.

Impaired GtH-II secretion could account for the restriction of 17,20βP production via reduced substrate availability since, in addition to 20β-HSD stimulation, GtH-II also acts to increase the activity of the enzyme systems cholesterol side-chain cleavage cytochrome P-450 ($P450_{SCC}$), 3β-hydroxysteroid dehydrogenase (3β-HSD) and $P450_{C17}$ which are responsible for thecal conversion of cholesterol to 17P via pregnenolone and progesterone (reviewed by Nagahama, 1997). In this context, during studies of testicular steroid production in male rainbow trout, Schulz et al. (1991, 1992) suggested that 20β-HSD might have a relatively low affinity for its substrate – 17P – and that, in turn, significant 17,20βP production would be highly dependent on a strong GtH-II stimulation to generate adequate precursor concentrations. Thus, while there may have been sufficient GtH-II to stimulate 20β-HSD synthesis in fish held at 16°C, the levels may have been insufficient to stimulate adequate 17P production for conversion to 17,20βP. Alternatively, reduced supply of 17P to the granulosa layer of the ovarian follicle might simply be accounted for by temperature impairment of enzyme activity or a reduction in cholesterol availability. In the latter case, holding temperature is known to influence lipid metabolism and deposition in fish (eg. Hazel, 1984; Ingemansson et al., 1993) and Jobling et al. (1995) reported a reduction in cholesterol content (11.0 – 8.7%) with increasing temperature (4 - 16°C) in Arctic charr oocytes sampled during vitellogenesis. Perhaps

more significantly, Liu and Stocco (1997) investigated heat shock-induced inhibition of steroidogenesis in mouse tumor cells and observed that the quantities and activities of both $P450_{SCC}$, and 3β-HSD were unaffected by heat shock, whereas synthesis of steroidogenic acute regulatory (StAR) protein was inhibited. StAR protein is understood to influence substrate supply to $P450_{SCC}$ by regulating cholesterol transfer between the outer and inner mitochondrial membranes, the acknowledged rate-limiting step in hormone-stimulated steroidogenesis (Liu and Stocco, 1997).

Irrespective of the likely mechanisms responsible for the low plasma steroid levels in female Atlantic salmon maintained at elevated temperatures, it is noteworthy that inhibition of ovulation was ongoing even in animals which displayed significant increases in plasma 17,20βP following administration of 17P alone (up to ~ 45 $ng.ml^{-1}$) or in combination with LHRHa (up to ~ 65 $ng.ml^{-1}$). Thus, it is apparent that low plasma 17,20βP is not solely responsible for the reproductive failure observed at elevated temperatures. This is not unexpected since, as discussed earlier, there is no evidence that 17,20βP directly stimulates ovulation in salmonids. Moreover, prior to considering the mechanism of ovulation, the presence of 17,20βP in the plasma need not imply successful completion of final oocyte maturation (FOM). In this context, additional aspects of the maturation process, down stream of MIS production, which could be temperature-influenced include;

1. Acquisition of ovarian maturational competence (OMC). Studies in a number of teleost species including the Atlantic croaker (*Micropogonias undulatus*) and the red seabream (*Pagrus major*) have revealed that MIS-mediated oocyte maturation cannot proceed without prior GtH-II-induced priming of the ovarian follicle (eg. Patiño and Thomas, 1990; Kagawa et al., 1994). This priming, or acquisition of OMC is characterised by increases in oocyte MIS receptor concentrations (Patiño and Thomas, 1990; Thomas and Patiño, 1991) and by increased cell-to-cell coupling (both within the granulosa layer and between granulosa cells and the oocyte) via gap junctions (GJ: Yoshizaki et al., 1995; Patiño and Kagawa, 1999). Both changes facilitate maturational signal transduction (Yoshizaki et al., 1995) and, in view of their GtH-II dependence, their susceptibility to impaired GtH-II production can be easily inferred.

However, with regard to oocyte maturation in salmonids, Pankhurst and Thomas (1998) observed greater *in vitro* advancement of maturational stage in oocytes from rainbow trout held at 18°C in response to 25-hydroxycholesterol (25-OHC) addition than in response to human chorionic gonadotropin (hCG) addition. Thus, Pankhurst and Thomas (1998) concluded that, at least for the stage of follicles examined, GtH-II-dependent acquisition of OMC was not a prerequisite for maturation in rainbow trout ovarian follicles.

2. Oocyte maturation promoting factor (MPF) production/action. Following receptor binding at the oocyte surface, the maturational action of 17,20βP is mediated by a cytoplasmic MPF (reviewed my Nagahama, 1997, 2000). The 17,20βP/receptor interaction stimulates *de novo* cyclin B synthesis via the translation of pre-existing cyclin B mRNA. Thereafter, cyclin B binds to cdc2 kinase and the resulting complex is activated by two progressive phosphorylation stages (Nagahama, 1997). Finally, the activated complex induces changes such as germinal vesicle breakdown (GVBD) and the resumption of meiosis (Nagahama, 1997, 2000). For the most part, the extent to which the above processes might be sensitive to temperature elevation is unclear. Nonetheless, it is recognised that temperature manipulation can be employed to disrupt the meiotic metaphase spindle during karyokinesis (Purdom, 1983), leading to the induction of triploidy in salmonid ova (eg. Lincoln and Scott, 1983; Benfey and Sutterlin, 1984, Bye and Lincoln, 1986). Thus, exposure to elevated temperature might also interfere with earlier aspects of oocyte meiotic development and MPF action. However, it should be noted that the temperatures employed for ploidy manipulation in salmonids typically exceed 25°C.

Even if FOM is complete, the potential for temperature-related effects to impair ovulation remains. As previously indicated, salmonid ovulation is probably mediated, at least in part, by GtH-II-stimulated $PGF_{2\alpha}$. However, the processes controlling ovulation are not well understood and may involve the cooperative action of numerous factors including proteases, protease inhibitors, progestational steroids, catecholamines and vasoactive peptides as well as eicosanoids (reviewed by Goetz and Garczynski, 1997). Accordingly, the likely effects of elevated temperature on the

processes controlling ovulation are unclear. Nonetheless, any impairment of GtH-II production might be expected to negatively influence ovulation. Furthermore, PG synthesis is dependent on the availability of free arachidonic acid (AA: Goetz, 1991; Hsu and Goetz, 1993). However, free AA is uncommon and AA is typically esterified and stored on phospholipid form (Hsu and Goetz, 1993). In view of the fact that temperature is known to markedly effect phospholipid biosynthesis in fish (Jobling et al., 1995), the previously highlighted effects of elevated holding temperature on lipid metabolism in fish might also be reflected in altered PG production.

Unfortunately, as GtH-II data are presently unavailable, the preceding discussion can be little more than speculative and it is impossible to determine with certainty whether the failure of fish held at 16°C to ovulate was due to impairment of FOM or ovulation. However, in this regard, it is noteworthy that Flett et al. (1996) reported low fertility in ovulated oocytes and the retention of ova in the ovarian stromal tissue of thermally compromised coho salmon. These authors suggested that the poor reproductive performance observed resulted from excessively early ovulation as a result of exposure to elevated temperatures. However, it could be argued that the retention of ova reported tends to indicate at least a partial failure to ovulate. Furthermore, the fact that the retained ova were reported to be over-ripe tends to imply that the oocytes had matured and that the reproductive failure was related to processes associated with ovulation. During the present study, ovarian biopsies were not taken from un-ovulated fish held at 16°C. Therefore, germinal vesicle position was unknown and the extent of maturational progression at 16°C could not be assessed.

Finally, consistent with earlier observations (Chapter 4), there was a differential pattern of survival in fish held at 16°C throughout. Sixty percent of controls survived to the end of the study whereas survival in fish which received only LHRHa was 30%. Only 10% of fish from each of the groups which received 17P survived to the completion of ovulation checks. Post mortem examinations were not conducted therefore, the cause or causes of death in these fish are unclear. Nonetheless, as all animals experienced identical handling protocols the differential mortality would appear to be treatment related. As previously discussed (Chapter 4), Dickhoff (1989) reported that LHRHa-treated Atlantic salmon consistently exhibited higher post-

spawning mortality than saline-injected controls or untreated animals. In a study conducted to examine the phenomenon more closely, mortality increased with increasing LHRHa dose and was exacerbated by exposure of fish to elevated water temperatures (13-15°C) (Dickhoff, 1989). As a result, Dickhoff (1989) concluded that these observations supported the role of GtH-II in mediating the programmed death of semelparous species. Following from this, the particularly high mortality of 17P-treated fish during the present study is interesting since Barry et al. (1995) showed that 17,20βP rapidly stimulated *in vitro* cortisol production by rainbow trout interrenal tissue via a cAMP-dependent mechanism. These authors hypothesised that regulation of corticosteroidogenesis might shift from the hypothalamic-pituitary-interrenal axis to the gonad at the time of spawning and related their findings to the known role of hypercortisolism in programmed death of salmonids discussed by Dickhoff (1989). With regard to the present study, analysis of cortisol levels in stored plasma samples may yield useful information relating the relative levels of 17,20βP and cortisol with individual survival.

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CHAPTER 6

EFFECT OF ELEVATED SUMMER TEMPERATURE ON STEROID PRODUCTION, VITELLOGENESIS AND EGG QUALITY IN TASMANIAN FEMALE ATLANTIC SALMON (*SALMO SALAR*)

6 EFFECT OF ELEVATED SUMMER TEMPERATURES ON STEROID PRODUCTION, VITELLOGENESIS AND EGG QUALITY IN TASMANIAN FEMALE ATLANTIC SALMON (*SALMO SALAR*).

6.1 Introduction

In female salmonids, reproductive development commences many months or even years prior to final oocyte maturation (FOM) and ovulation (Billard, 1985). Vitellogenesis, the phase of rapid ovarian growth which is characterised by the hepatic production and ovarian uptake of the yolk protein precursor vitellogenin (Vtg) (reviewed by Specker and Sullivan, 1994), in turn occurs over several months of the female reproductive cycle (Billard, 1985). During vitellogenesis, individual oocytes may increase in size by several hundred-fold and Vtg can account for over 90% of final oocyte volume (reviewed by Tyler, 1991; Tyler et al., 2000). Since salmonids are generally recognised as autumn and winter spawners (reviewed by Scott, 1990), much of the vitellogenic oocyte growth takes place during summer and early autumn (eg. Bromage and Cumaranatunga, 1988; Tyler et al., 1990; Estay et al., 1998; Chapter 2) when natural water temperatures tend to be elevated.

In fish, many aspects of reproductive development, including spermatogenesis, oogenesis, spermiation and ovulation, are known to be significantly influenced by environmental temperature (reviewed by Van Der Kraak and Pankhurst, 1997). In relation to the effects of upper extremes of temperature in salmonids, it has been demonstrated that maintenance at elevated temperature can inhibit FOM and ovulation in Arctic charr (*Salvelinus alpinus*) (Gillet, 1991), Atlantic salmon (*Salmo salar*) (Taranger and Hansen, 1993; Chapter 3, 4 and 5) and rainbow trout (*Oncorhynchus mykiss*) (Pankurst et al., 1996; Pankhurst and Thomas, 1998). In contrast, the effects of chronically-elevated environmental temperature on vitellogenesis are poorly understood. Exposure of Atlantic salmon post-smolts to 3°C reduced the hepatic vitellogenic output relative to that observed at 10°C (Korsgaard et al., 1986). Similarly, MacKay and Lazier (1993) reported a progressive increase in the rate and extent of Vtg production by rainbow trout held at 4, 9 and 15°C. Pankhurst et al. (1996) reported essentially unimpaired estrogen synthesis (and, by

inference, vitellogenesis) in Tasmanian rainbow trout reared at temperatures as high as 21°C but Chmilevsky (2000) briefly described how gonadal development was arrested in the same species following exposure to 22-23°C. Thus, it is unclear at which point the apparent stimulatory effect of elevated temperature on vitellogenesis becomes an inhibitory one.

There is limited information on the effects of temperature change on vitellogenesis in non-salmonids. In the common wolffish (*Anarhichas lupus*), Tveiten and Johnsen (2001) showed that exposure to elevated water temperatures (12°C) during vitellogenesis delayed ovarian steroidogenesis, although effects on vitellogenesis, inferred from differences in egg size, were less clear (Tveiten and Johnsen, 1999).

In female Atlantic salmon, impairment of ovarian steroidogenesis was also apparent during the late stages of vitellogenesis in fish held at elevated temperature (Chapter 3). Plasma levels of both 17β-estradiol (E_2) and its precursor testosterone (T) were significantly reduced in fish held at 16°C relative to levels observed in fish held at 6 and 11°C (Chapter 3). Importantly, since the primary function of E_2 is the stimulation of hepatic synthesis and ovarian sequestration of Vtg (Specker and Sullivan, 1994), it was concluded that the reduced viability observed in ova from fish maintained at 16°C might have resulted in part from impairment of vitellogenesis (Chapter 3). If this is the case, then it seems likely that the higher temperatures experienced by Tasmanian fish earlier in the summer (summer and early autumn water temperatures in Tasmania routinely exceed 19-20°C) might also exert inhibitory effects early in vitellogenesis. Accordingly, the present study was conducted in order to assess the effect of temperature manipulation on vitellogenesis and egg and larval quality in Tasmanian female Atlantic salmon exposed from the early stages of vitellogenesis. Groups of female Atlantic salmon were maintained at temperatures of 14, 18 and 22°C for 3 months from mid-summer onwards. At the end of the exposure period, temperatures were reduced to 8°C to facilitate FOM, ovulation and subsequent egg incubation. Blood samples were collected at regular intervals during the period of temperature manipulation, and plasma levels of E_2, T, cortisol and Vtg were measured to assess the relationship between endocrine status and Vtg production, and differences in reproductive development and gamete quality.

6.2 Materials & Methods

6.2.i Husbandry, Temperature and Photoperiod Regimes

Ninety sexually maturing 2+ female Atlantic salmon (mean weight 4.6kg) were transported from Aquatas Pty. Ltd., (Margate, Tasmania) to SALTAS Freshwater Operations (Wayatinah, Tasmania) in mid November 1998 and were maintained in a single circular 44m^3 outdoor tank supplied with river water (~ 15 $l.s^{-1}$) at natural temperature (~ 13-15°C at time of transfer). Fish were fed to satiation on a commercial broodstock ration (Pivot Aquaculture Ltd., Cambridge, Tasmania). In mid January 1999, fish were transferred from natural conditions to 9 temperature-controlled 4m^3 Rathbun tanks (10 fish per tank) initially set at a water temperature of 14°C. Tanks were supplied with partially recirculated water. Biological filtration and water exchanges of 50% per day were employed to prevent the accumulation of toxic metabolites. Oxygen saturation was maintained at 100-120% by the addition of gaseous oxygen via ceramic diffusers. Twenty-four hours after transfer, the temperature of 3 tanks was increased to 18°C and that of a second group of 3 tanks to 22°C at a maximum rate of 1°C.day^{-1}. Three tanks were held at 14°C. Following transfer to temperature-controlled conditions, fish were unfed and exposed to a simulated natural photoperiod (42° S). In order to facilitate final oocyte maturation and ovulation, the water temperatures of all three systems were reduced in mid April 1999, at a maximum rate of 2°C.day^{-1}, such that all three systems reached a temperature of 8°C on April 20, the date by which fish under natural conditions would have been expected to have completed vitellogenesis (Chapter 3).

6.2.ii Fish sampling and handling

Twenty-four hours after the temperature-controlled systems had reached the correct holding temperatures of 14, 18 or 22°C on 19 January, all fish from each of the three temperatures were anaesthetised (25p.p.m. Aqui-s, Crop & Food, New Zealand) and tagged by placing visible implant tags (VI Tags, Northwest Marine Technology Inc, Shaw Island, WA) in the left adipose eyelid according to the methods described by Bergman et al. (1992) and Kincaid and Calkins (1992). Blood samples were taken from each fish by puncture of the duct of Cuvier (Lied et al., 1975) using heparinized (lithium heparin) syringes and 22G needles. After centrifugation, the resulting plasma

was stored at -20°C prior to analysis of steroid hormone and vitellogenin levels. Anaesthesia and blood sampling was repeated at 3 week intervals until mid April 1999. Ovulation checks were commenced at the start of the recognised spawning season (late April) and continued at 3-4 day intervals until all surviving fish had ovulated.

6.2.iii Ova Fertilisation and Incubation

Fish that expressed ova in response to the gentle application of pressure to the abdomen were transferred to a holding tank maintained at 8°C. After 24h at 8°C fish were killed by a blow to the head, towelled dry and ova were expressed into a stainless steel sieve. In order to facilitate ova collection, a 2cm cut was made at the genital papilla. Ova were transferred to a stainless steel bowl and fertilised using pooled milt from 3-4 naturally spermiating males. Ova and milt were gently mixed and water (500ml) was added to ensure sperm activation. After 2 mins, ova were rinsed with clean water then left to water-harden for 60 mins. After water-hardening, sample batches (1000-1500) of ova from each female were removed and incubated at 8°C in mesh baskets (4.0cm x 10.0cm x 12.0cm) placed in Heath vertical incubator trays (Marisource Inc., Tacoma, WA). Sub-samples of ova from each female were collected after water-hardening and fertilisation (%) was determined on the basis of first cell division after 120 degree-hours, visualised by treatment with a clearing solution (1:1:1 v/v methanol:acetic acid:water) for 2 mins. The diameters of 10 cleared oocytes from each female were measured (± 0.01 mm) using digital vernier callipers and individual mean oocyte diameters were used to calculate a grand mean oocyte diameter for each temperature regime. Eyed ova survival (%) was observed directly after 250 degree-days.

6.2.iv Plasma Hormone and Vtg Measurement

Plasma Steroids

Plasma levels of E_2, T and cortisol were measured by radioimmunoassay following extraction with ethyl acetate using the reagents and protocols described by Pankhurst and Carragher (1992). Extraction efficiency (mean recovery of ^{3}H-labelled steroid from triplicates of a plasma pool) was 59.2-66.5, 85.3-90.6% and 94.3-100% for E_2, T and cortisol respectively and values for each steroid were adjusted accordingly.

Interassay variability measured using aliquots of a pooled internal standard was (%CV(n)) 12.4(10) and 10.8(10), 12.2(8) for E_2, T and cortisol respectively.

Plasma Vtg

Induction, Purification and Partial Characterization of Atlantic Salmon Vtg (AS-Vtg)

Four 3-year old male Atlantic salmon (mean weight ~ 5.0 kg) were separated into two treatment groups (n = 2) and injected (ip) with either saline or a saline suspension of E_2 at a dose of 2 $mg.kg^{-1}$. The injection volume was 1 ml and treatment was repeated after an interval of 7 days. After a further 7 days, fish were anaesthetised and bled as previously described. After centrifugation, a saturated solution of the serine protease inhibitor phenylmethyl-sulfonylflouride (PMSF, sigma) was added to plasma samples (2:1 v:v plasma:PMSF) to prevent proteolysis, and the resulting solution was stored at -20°C prior to transfer to the laboratory for Vtg purification.

After thawing, 2 ml of sample (plasma/PMSF) were applied onto a Sephaceryl HR 300 (Amersham Pharmacia Biotech AB) 900 x 16 mm gel filtration column and eluted with 0.05 M Tris base – 0.15 M NaCl (pH 8.0), at a flow rate of 10 $ml.h^{-1}$. Fractions (volume 2 ml) were collected and following transfer to quartz cuvettes, fraction absorbance at 280 nm was read using a UNICAM 8625 UV/VIS spectrophotometer.

Putative Vtg peak fractions (identified on the basis of their presence in E_2- but not saline-injected fish) obtained from gel filtration chromatography were further purified by SDS-PAGE (4% stacking acrylamide and 10% resolving acrylamide) performed according to Laemmli (1970). Protein components were visualized by staining using 0.025% Coomassie Brilliant Blue R-250 (CBB).

Production of Anti-AS-Vtg Serum (abAS-Vtg)

Protein bands identified as putative Vtg were excised from the SDS-PAGE and de-stained using 50% methanol and 10% acetic acid (1h) followed by 5% methanol and 7% acetic acid (1h). Gel pieces were washed with several changes of sterile distilled water then homogenized in 4 ml phospate-buffered saline (PBS). Homogenates were diluted 1:1 with Montanide adjuvant and divided to yield 8 x 1 ml inocula (~ 50 $\mu g.ml^{-1}$ purified Vtg). Two sheep received a total of 4 inoculations over a 2-month

period and blood was collected at the time of the final inoculation and after a further 7-28 days. A total of ~ 1.5 l abAS-Vtg was obtained.

Western Blot Analysis

Proteins were electrotransferred from SDS PAGE onto a 45 μm nitro-cellulose membrane (Biorad) using a Hoefer Semi-Phor Semi-dry transfer unit at 0.8 $mA.cm^{-2}$ for 2 h. Transfer buffer contained 48 mM Tris, 39 mM glycine, 20% methanol (pH 9.2) (Schaefer and Bjerum-Neilsen transfer buffer). After transfer, the nitro-cellulose membrane was stained in Ponceau Red to confirm transfer, de-stained in water and incubated overnight with 20 mM Tris-HCl, 0.5 M NaCl, (pH 7·5) (TBS) plus 5% powdered skimmed milk (TBSB) to block the free binding sites. Following blocking, the membrane was cut into strips and transferred to separate containers. Membrane strips were rinsed with TBS, then abAS-Vtg (diluted 1:100 in TBS containing 3% skim milk: TBSD) was added and incubated for 1 h. The same dilution of pre-immune sheep serum was used as a negative antibody control. Strips were washed 3 times over a 15 min period in TBS, TBS containing 0·01% Tween-20 (TBST) and TBS, then incubated for 1 h with the secondary antibody (Donkey Anti-Sheep IgG alkaline phosphatase conjugate: DASC-AP, Sigma) diluted 1:10,000 in TBSD. The resulting complex was detected with bromo-4-chloro-3-indolyl-phosphate and dimethylformamide nitrobluetetrazolium chloride (BCIP/NBT) (Biorad) and the nitro-cellulose strips were rinsed with distilled water and dried. All incubations were conducted at laboratory ambient temperature.

AS-Vtg Enzyme-linked Immunosorbent Assay (ELISA) Procedure

The optimum abAS-Vtg concentration for use during ELISA was established using a chequer-board procedure as follows:

One hundred microlitre volumes of purified AS-Vtg were serially diluted in 50 mM carbonate buffer (pH 9.6), from 20 μg to 0.018 $\mu g.ml^{-1}$ in a microtitre plate. Dilutions were made across the plate giving a total of 8 antigen dilution series and the plate was incubated overnight, on a shaker, at 4°C. The plate was then washed 4 times on a Biorad automatic plate-washer with 300 μl of TBS. After washing, all wells were blocked for 1 h at 37°C with 300 μl per well of TBSB. Following blocking, wells

were emptied and 100 µl of abAS-Vtg was serially diluted in TBSD from 1/100 to 1/12800, down the plate such that each row represented a different antibody dilution across a range of serially diluted antigen concentrations. The plate was incubated for 1 h at 37°C and then washed 4 times as before. After washing, 100 µl of freshly prepared 1mg.ml^{-1} 4-nitrophenylphosphate (pNPP) in diethanolamine $MgCl_2$ buffer (Sigma) was added to each well and absorbance was read at 405 nm, using a Tecan plate-reader, after incubation at 37°C for 30 min.

Subsequently, using a procedure developed according to the general methods described by Crowther (1995), plasma Vtg levels were measured by ELISA as follows:

A 96-well microtitre plate was coated with 100 µl of 2.5 µg ml^{-1} purified AS-Vtg in 50 mM carbonate buffer (pH 9·6). The plate was then incubated overnight at 4°C and washed 4 times with TBST (300µl per well). Thereafter, the plate was blocked by incubation at 37°C for 1 h with TBSB (300µl per well). Plasma samples (diluted 1:250 in TBSD) or Vtg standards were added to a second microtitre plate (100µl per well). A 1:500 dilution of the abAS-Vtg in TBSD was then added to the plate at 100µl per well (excluding non-specific binding wells). The competition plate was then incubated overnight with shaking at 4°C. One hundred microlitres of the competition solution were then transferred to each corresponding well of the first plate, incubated for 1 h at 37°C, and washed 4 times with TBST (300µl per well). A 1:10,000 dilution of DASC-AP in TBSD was then added (100µl per well), the plate incubated for 1 h at 37°C and washed as before. Finally, 100µl per well of freshly prepared 1 mg.ml^{-1} pNPP in diethanolamine $MgCl_2$ buffer (Sigma) was added as substrate and colourimetric measurement was made at 405 nm as previously described.

6.2.v Chorion Morphology

At stripping, a sub-sample (N=10-20) of unfertilised ova from each ovulated female was placed in Bouin's fixative and after 48h, fixed ova were transferred to 70% ethanol. Fixed ova stored in 70% ethanol were cut into quarters with a scalpel blade, and the chorion from one of the quarters was dehydrated in 90% ethanol for 30 mins, and then 100% ethanol for 30 mins, prior to critical point drying in a BAL-TEC CPD-

030 critical point dryer. The dried samples were fixed to scanning electron microscope (SEM) stubs (5 samples per stub) with double-sided carbon tape to allow exposure of the outer surface of the chorion to the electron beam. The stubs were then coated with gold in a Union FL9496 Blazer splutter coater and chorion samples were observed at approximately 150x magnification using an Electro Scan Environmental SEM. The appearance of the chorion and in particular the presence or absence of holes, was recorded and representative electron micrographs were taken to provide a visual record of chorion external morphology.

6.2.vi Statistical Analysis

Where appropriate, steroid, Vtg and egg quality data were analysed by one-way and two-way ANOVA, and Tukey's HSD test using the SYSTAT 8.0 for Windows computer package. The same package was used to derive linear correlation coefficients between parameters and to construct contingency tables for the comparison of treatment-related differences in egg external morphology. Proportion data were normalised by arcsin transformation.

6.3 Results

6.3.i Ovulation

Ovulations in fish from all three temperature regimes commenced simultaneously on 18 May (Figure 6.1). The 14 and 22°C treatment groups completed ovulation on 6 June, whereas fish from the 18°C treatment did not complete ovulation for a further 8 days. A level of 80% ovulation was first exceeded by fish from the 22°C treatment on 24 May, whereas similar progress was only achieved by the 14 and 18°C treatment groups after a further 7 and 17 days respectively.

6.3.ii Ova Size and Viability

The mean diameter of ova from fish from the 14 and 18°C temperature treatments were not significantly different (~ 5.7 mm, Figure 6.2). However, ova from fish which had been held at 22°C were significantly ($P < 0.001$) smaller (~ 5.4 mm) than those from fish held at 14 and 18°C. Similar differences and levels of significance were observed for mean fertility ($P < 0.01$, Figure 6.3) and mean survival of ova to the eyed

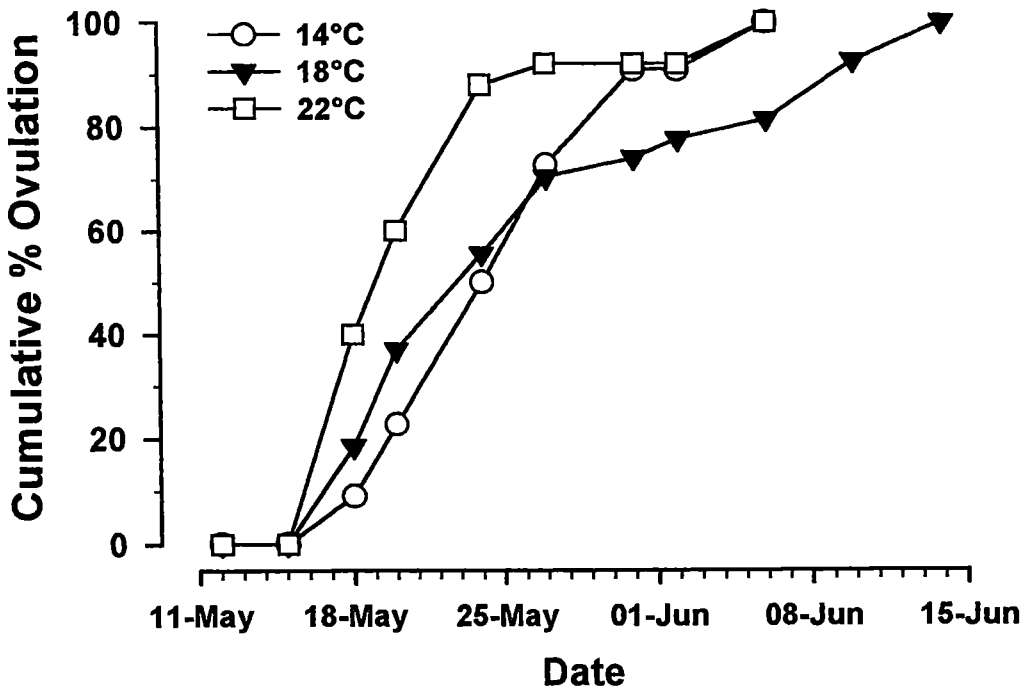

Figure 6.1 Cumulative percent ovulation in female Atlantic salmon maintained at 14°C (○), 18°C (▼), or 22°C (□).

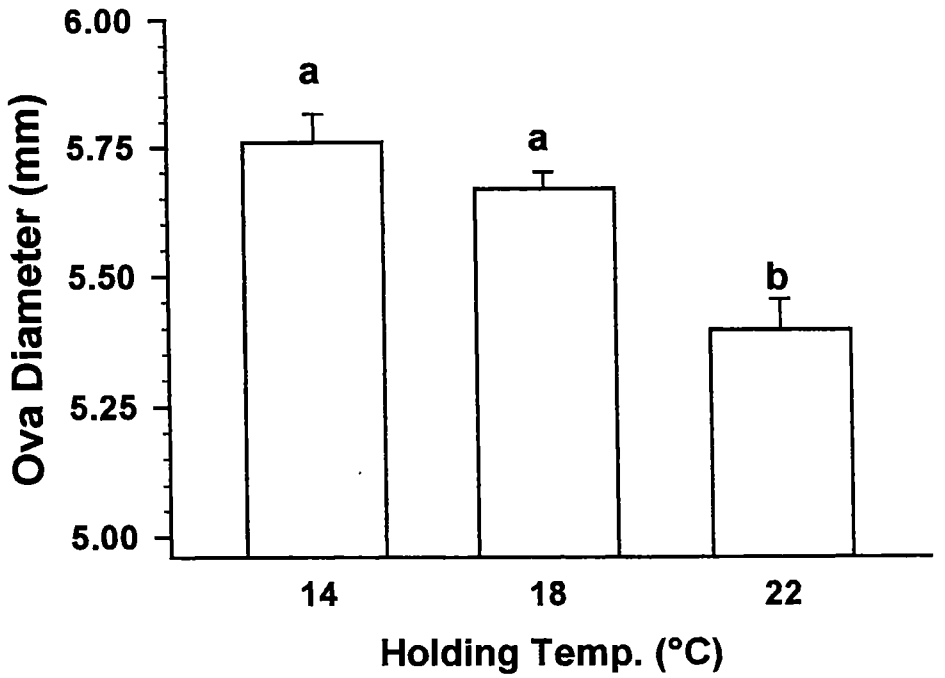

Figure 6.2 Mean (+ S.E.M.) diameter (mm) of ova from female Atlantic salmon maintained at 14, 18, or 22°C. Bars with the same superscript are not significantly different (P>0.05).

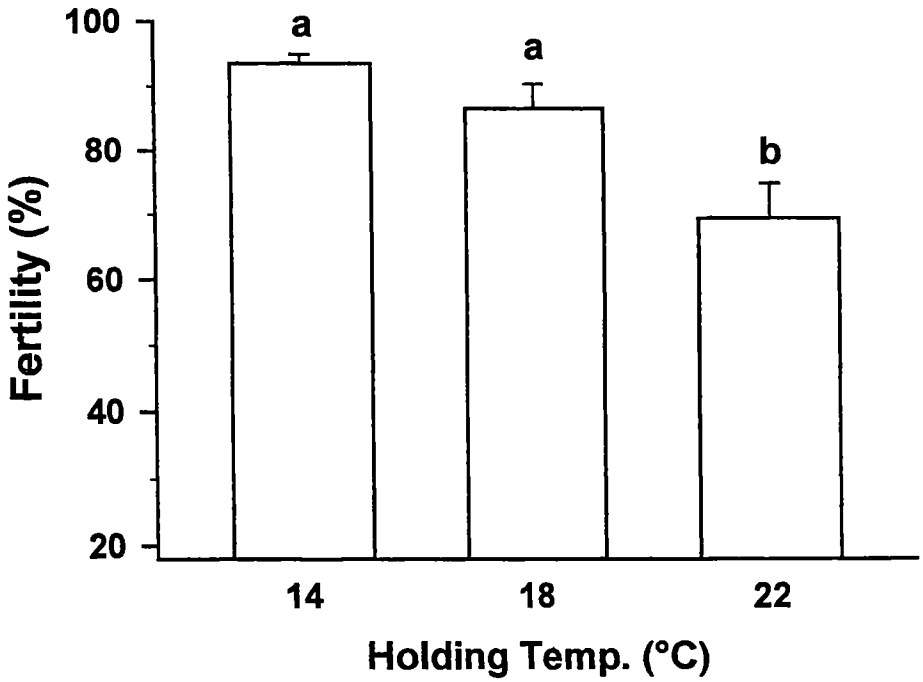

Figure 6.3 Mean (+ S.E.M.) percent fertility of ova from female Atlantic salmon maintained at 14, 18, or 22°C. Bars with the same superscript are not significantly different (P>0.05).

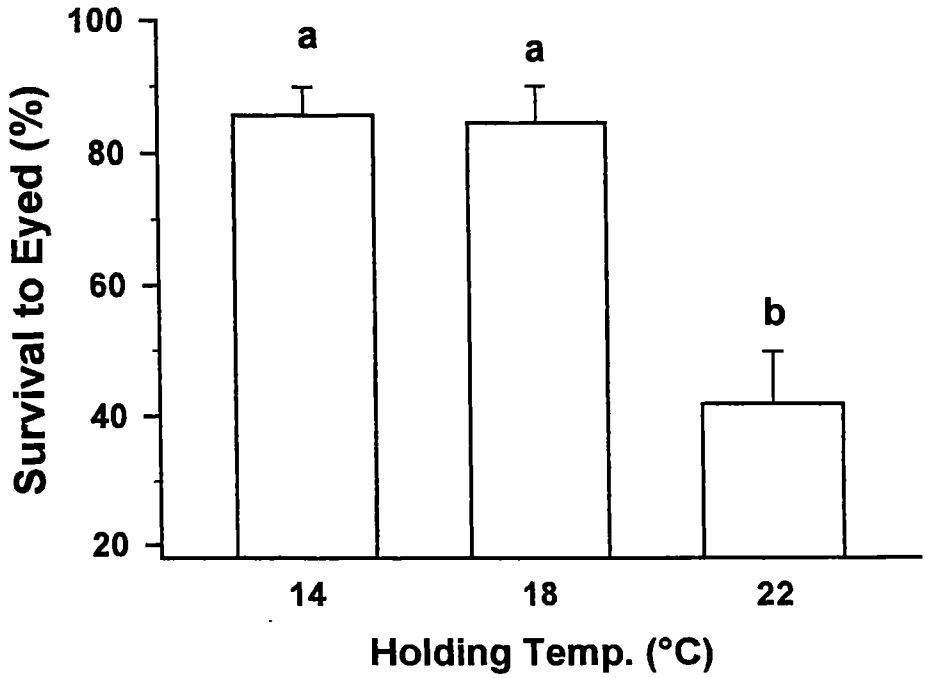

Figure 6.4 Mean (+ S.E.M.) percent survival to the eyed stage of ova from female Atlantic salmon maintained at 14, 18, or 22°C. Bars with the same superscript are not significantly different (P>0.05).

stage ($P < 0.001$, Figure 6.4). Mean fertility exceeded 85% in ova from fish which had been held at 14 and 18°C but was below 70% in ova from fish maintained at 22°C. Similarly, mean eyed-egg survival was greater than 80% in ova from fish held at lower temperatures, whereas ova from fish held at 22°C displayed a mean survival of only ~ 42%.

6.3.iii Plasma Steroids

Mean plasma E_2 levels were below 3 $ng.ml^{-1}$ at the commencement of sampling and increased to exceed 24 $ng.ml^{-1}$ in all treatments at the completion of sampling in mid April (Figure 6.5). Plasma E_2 in fish held at 22°C was significantly lower than that in fish held at 14 or 18°C at the first two sample points ($P < 0.005$ and 0.001 respectively). At the third sample time there were no significant differences and at the fourth sample point, plasma E_2 levels at 14 and 22°C were significantly different ($P < 0.001$). At that time, the differences between E_2 levels at 18°C and those at 14 and 22°C also approached significance ($P = 0.065$ and 0.054 respectively). E_2 levels in the final samples (mid April) were significantly lower at 18 and 22°C relative to those in fish held at 14°C ($P = 0.021$ and 0.017 respectively).

Mean plasma T levels were below 4 $ng.ml^{-1}$ in mid January and increased to > 14 $ng.ml^{-1}$ in all treatments at the completion of sampling (Figure 6.6). At the first sample point, plasma T levels were significantly higher in fish held at 18°C relative to fish held at both 14 and 22°C ($P = 0.009$ and 0.007 respectively). At the second sample, there was no difference between treatments, whereas at the third sample time fish held at 22°C had significantly higher T levels than fish held at either 14 or 18°C ($P = 0.014$ and 0.019 respectively). There were no differences between treatments at the time of the fourth sample ($P = 0.099$), but at the final sample, plasma T levels were significantly lower in fish held at 18 and 22°C than in fish maintained at 14°C ($P < 0.001$).

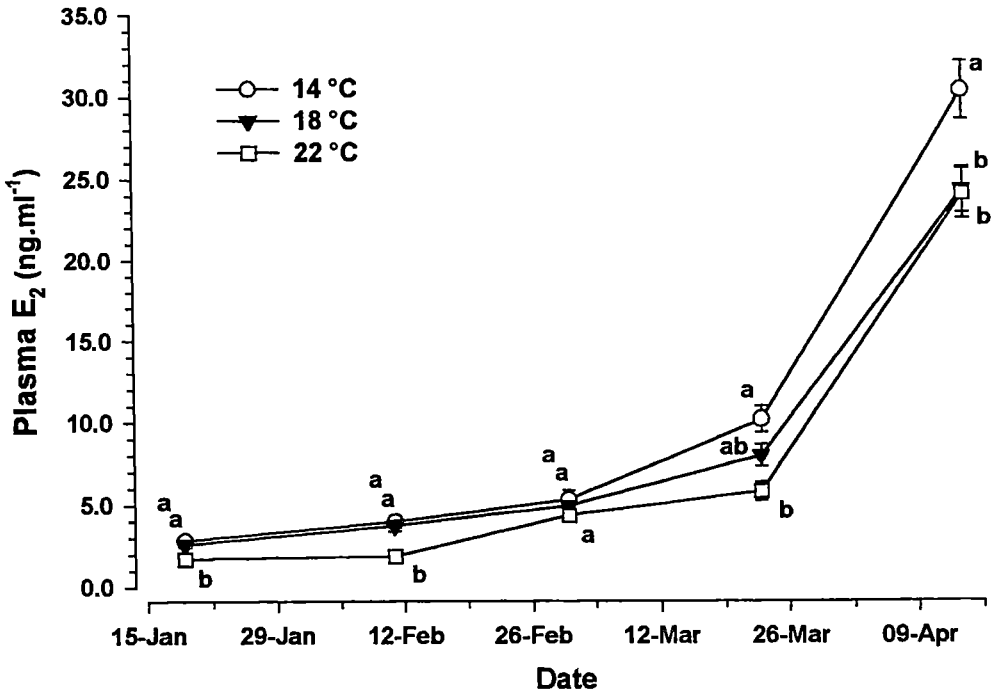

Figure 6.5 Mean (± S.E.M.) plasma 17β-estradiol (E_2, ng.ml^{-1}) in female Atlantic salmon maintained at 14°C (○), 18°C (▼), or 22°C (□). Data points with the same superscript are not significantly different (P>0.05), n = 23-29.

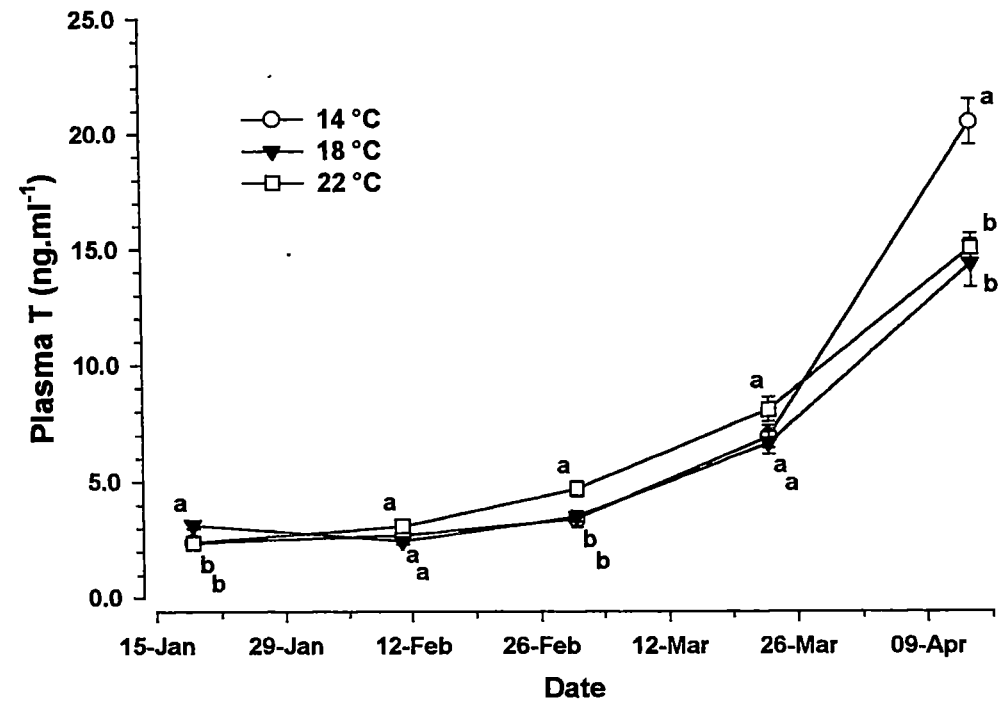

Figure 6.6 Mean (± S.E.M.) plasma testosterone (T, ng.ml^{-1}) in female Atlantic salmon maintained at 14°C (○), 18°C (▼), or 22°C (□). Data points with the same superscript are not significantly different (P>0.05), n = 23-29.

At the commencement of sampling, plasma cortisol levels were significantly higher (> 100 ng.ml^{-1}) in fish held at 22°C than those in fish held at 18°C (> 70 ng.ml^{-1}; $P < 0.001$) which were, in turn significantly higher than those in fish held at 14°C (< 60 ng.ml^{-1}; $P < 0.05$) (Figure 6.7). At the second sample point, plasma cortisol levels were also significantly different among all treatments (18°C > 22°C > 14°C; $P < 0.05$), and at the third sample, plasma cortisol levels were significantly lower in fish held at 14 and 18°C than in fish maintained at 22°C ($P < 0.01$ and $P < 0.005$, respectively). At the fourth sample, plasma cortisol levels were again significantly different among all treatments (18°C > 22°C > 14°C; $P < 0.05$), while, at the final sample point, fish held at 14°C had significantly lower cortisol levels than fish held at 18°C ($P < 0.05$) but levels in fish held at 22°C did not differ from those at 14 or 18°C.

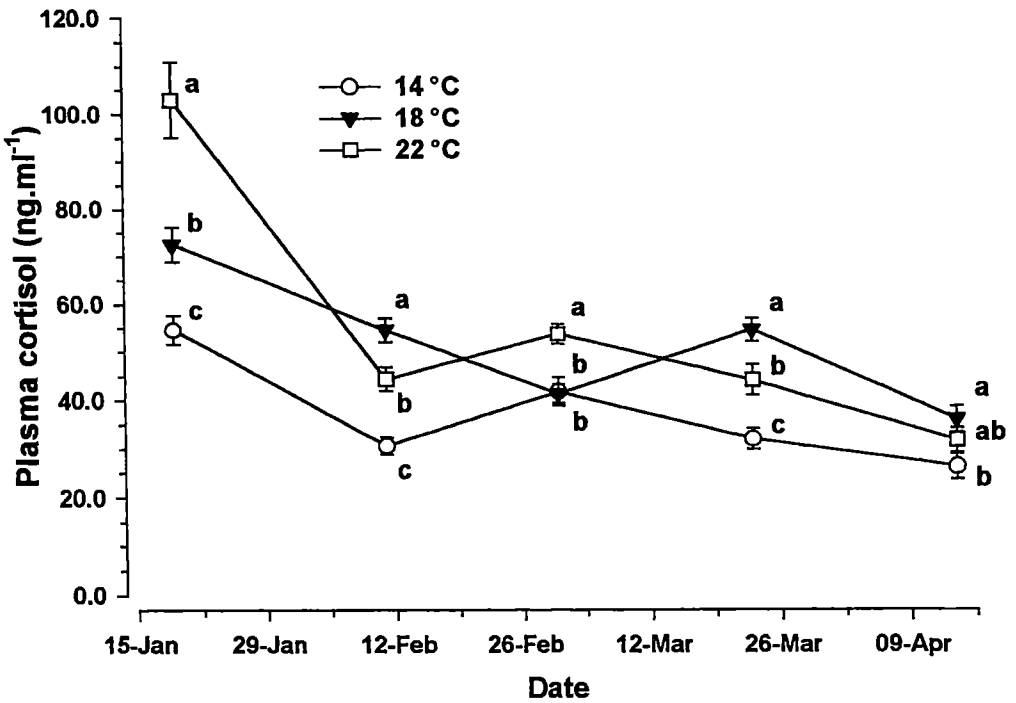

Figure 6.7 Mean (± S.E.M.) plasma cortisol (ng.ml^{-1}) in female Atlantic salmon maintained at 14°C (○), 18°C (▼), or 22°C (□). Data points with the same superscript are not significantly different ($P>0.05$), n = 23-29.

6.3.iv Plasma Vtg

Under gel filtration, a protein peak eluting at fraction 20 was observed in the plasma from each E_2-treated male fish but a similar peak was absent in both control animals (Figure 6.8). Comparison with a range of protein standards indicated an apparent molecular weight (MW) of ~ 540 kD.

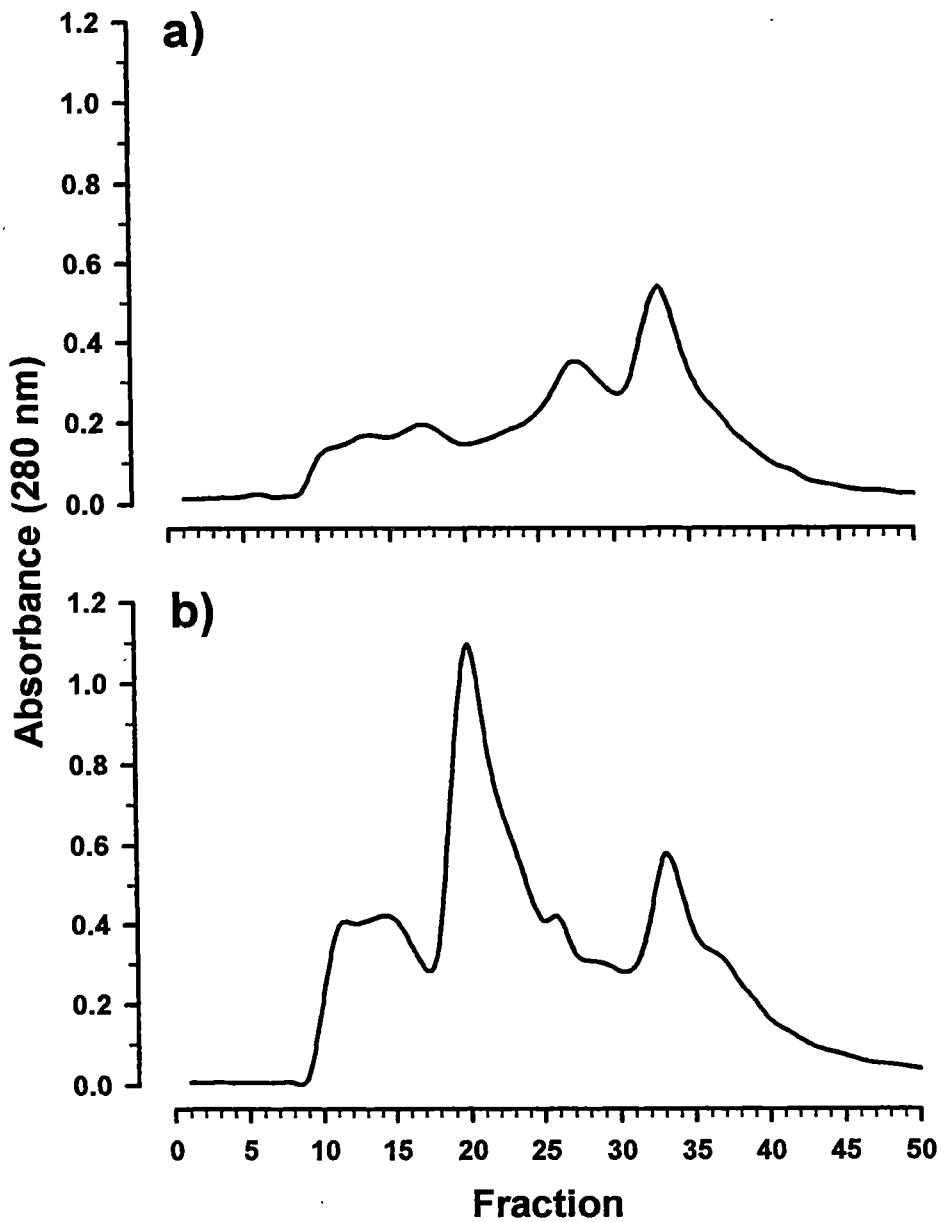

Figure 6.8 Elution profiles from gel filtration chromatography of plasma from a) saline-injected and b) E_2-injected male Atlantic salmon displaying a putative vitellogenin (Vtg) peak at fraction 20.

Following abAS-Vtg production where a total of ~ 1.5 l of abAS-Vtg was obtained, Western Blot analysis confirmed that the abAS-Vtg exhibited a high specificity to the putative AS-Vtg purified from the plasma of E_2-treated male fish.

The ELISA for AS-Vtg exhibited competitive binding for Vtg standards where displacement was linear across the dilution range and where plasma from E_2-treated males diluted out in parallel to Vtg standards, whereas plasma from control fish exhibited no cross-reactivity (Figure 6.9). Interassay variability measured using aliquots of a pooled internal standard was (%CV(n)) 27.4(20).

Mean plasma Vtg levels in fish held at 14°C ranged between ~9.5 and 16 $mg.ml^{-1}$, while at 18°C, plasma Vtg levels ranged from ~6 to 13 $mg.ml^{-1}$ (Figure 6.10). In contrast, plasma Vtg in fish held at 22°C increased from approximately 6 $mg.ml^{-1}$ at the commencement of sampling to approximately 29 $mg.ml^{-1}$ at the completion of sampling (Figure 6.10). At the first sample point, plasma Vtg levels were significantly different among all treatments (14°C > 18°C > 22°C; $P < 0.05$), whereas, at the second sample, plasma Vtg levels were significantly lower in fish held at 18 and 22°C than in fish maintained at 14°C ($P < 0.05$ and $P < 0.01$ respectively). At the third sample, fish held at 14°C had significantly higher Vtg levels than fish held at 22°C ($P < 0.01$) but levels in fish held at 18°C did not differ from those at 14 or 22°C whereas, at the fourth sample, fish held at 14°C had significantly higher Vtg levels than fish held at 18°C ($P < 0.01$) but levels in fish held at 22°C did not differ from those at 14 or 18°C. In contrast, at the final sample, plasma Vtg levels were not significantly different in fish held at 14 and 18°C but levels in fish held at 22°C showed a marked and significant ($P < 0.001$) increase.

Comparison of E_2, Vtg and cortisol data revealed that, plasma Vtg levels displayed a weak but significant positive correlation with plasma E_2 levels at the first ($R = 0.42$, Bartlett χ^2-Statistic = 16.7, d.f. = 1, $P < 0.001$) and second samples ($R = 0.33$, Bartlett χ^2-Statistic = 8.8, d.f. = 1, $P < 0.01$). Similarly, plasma Vtg levels displayed a weak but significant negative correlation with plasma cortisol levels at the first ($R = -0.48$, Bartlett χ^2-Statistic = 19.8, d.f. = 1, $P < 0.001$) and second samples ($R = -0.44$, Bartlett χ^2-Statistic = 16.1, d.f. = 1, $P < 0.001$) but there was no significant relationship between plasma E_2 and cortisol levels at any sample time.

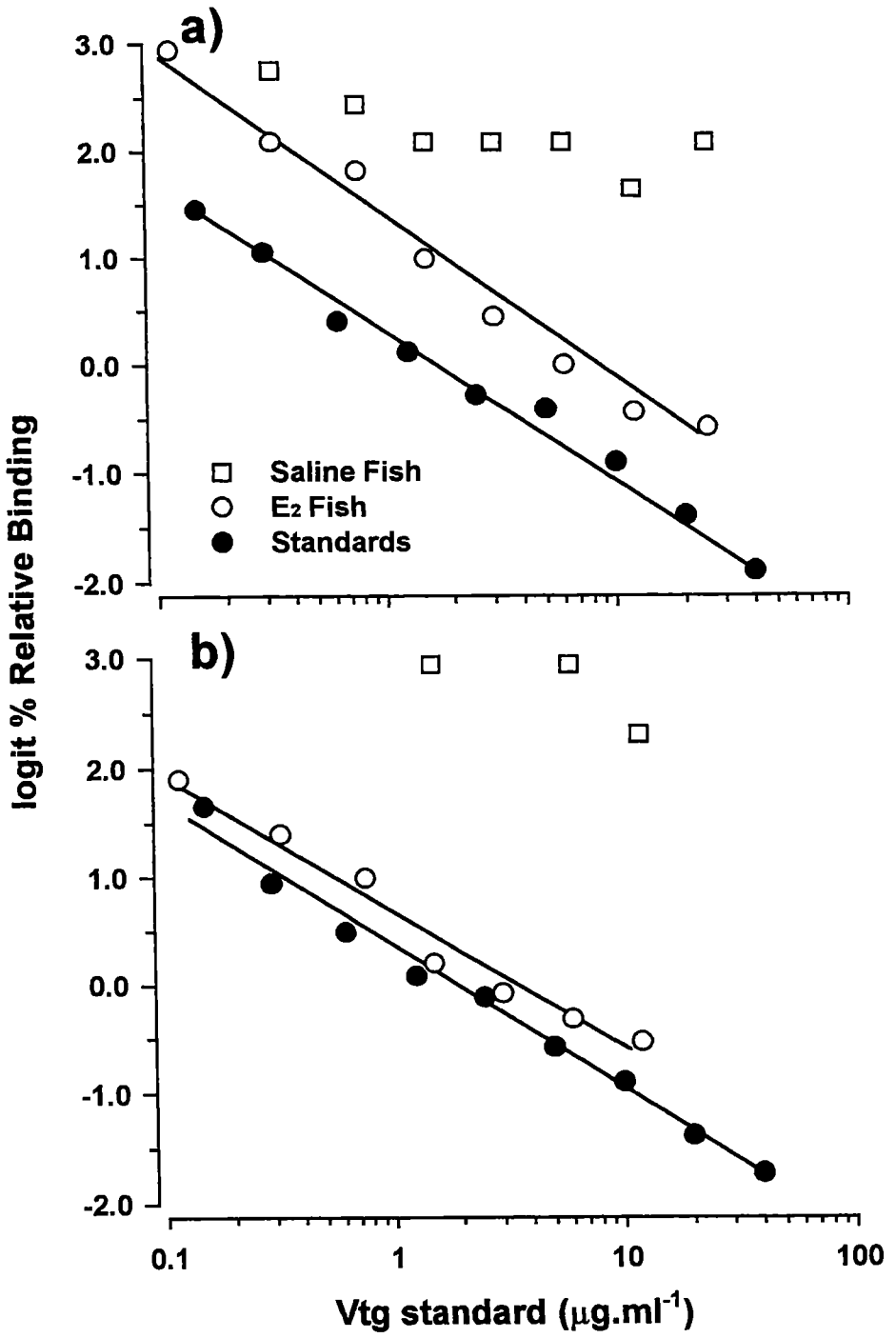

Figure 6.9 Logit/log transformation of competitive binding curves of a) plasma from saline-treated fish 1 (□), E2-treated fish 1 (○) and purified As-Vtg (●) and b) plasma from saline-treated fish 2 (□), E2-treated fish 2 (○) and purified As-Vtg (●).

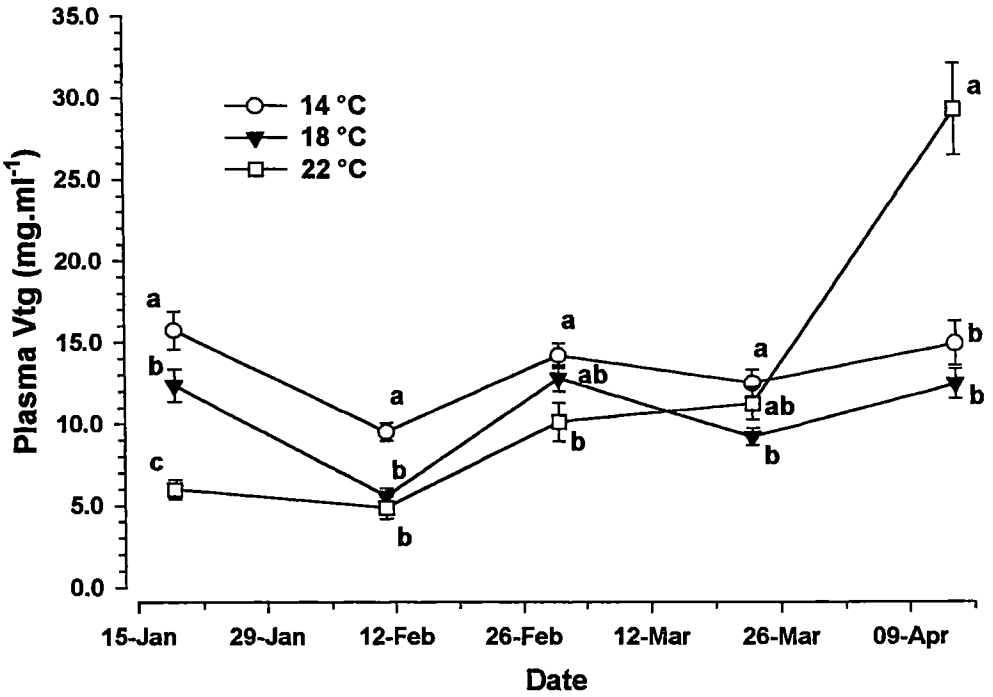

Figure 6.10 Mean (± S.E.M.) plasma vitellogenin (Vtg, ng.ml^{-1}) in female Atlantic salmon maintained at 14°C (○), 18°C (▼), or 22°C (□). Data points with the same superscript are not significantly different (P>0.05), n = 23-29.

6.3.v Chorion Morphology

Examination of chorion morphology revealed a number of differing egg surface characteristics. The majority of samples exhibited a chorion surface where the plugs sealing the pore channels which had been the conduit for maternal investment between the granulosa layer of the ovarian follicle and the developing oocyte were clearly visible (Figure 6.11a and b). The open micropyle of the ovum was also visible in a number of samples (Figure 6.11c). However, in addition to the normal features above, holes were observed in the chorion of many samples (Figure 6.11d). These holes appeared to have originated from blister-like eruptions on the surface of the chorion such that they resembled raised craters of 2-10 μm in diameter (Figure 6.11d). Closer examination revealed that the holes were irregular in diameter and that they were partially obstructed by fibrous material (Figure 6.11e).

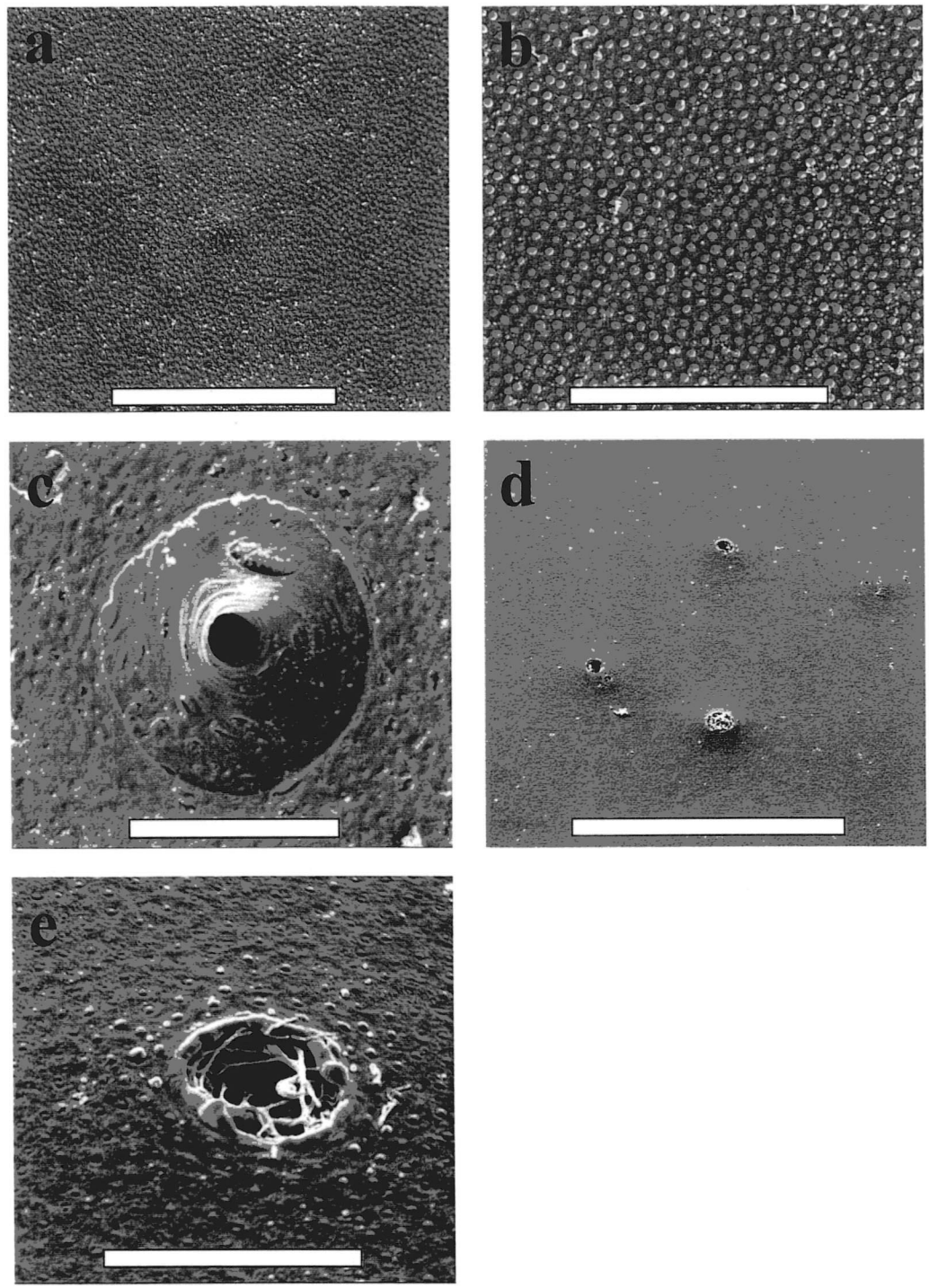

Figure 6.11 Scanning electron micrographs of (a) & (b) the surface of a typical Atlantic salmon ovum displaying protein plugs sealing the pore channels of the zona radiata (scale bar = 40μm and 25μm respectively), (c) the micropyle of a typical Atlantic salmon ovum (scale bar = 15μm), (d) a damaged Atlantic salmon ovum displaying blister-like holes in the chorion (scale bar = 150μm) and (e) a damaged Atlantic salmon ovum displaying the detail of a blister-like hole in the chorion (scale bar = 15μm).

Among fish maintained at 14°C, 27.3% of females produced ova that contained abnormal holes (Table 6.1) and the mean incidence of holes in those egg batches was 32.6% such that the overall prevalence of damaged ova from fish held at 14°C was 8.9%. At 18°C, 60.9% of females produced ova with holes and the mean incidence of holes was 31.9% (Table 6.1), yielding an overall prevalence of damaged ova of 19.4%, while at 22°C, 38.5% of females produced ova with holes, with a mean hole incidence of 38.4% (Table 6.1) such that the overall prevalence of damaged ova was 14.8%. The proportion of females which produced damaged ova was significantly higher at 18°C than at 14°C (Pearson χ^2-Statistic = 5.14, d.f. = 1, $P < 0.05$), whereas, the proportion of females at 22°C which produced damaged ova did not differ significantly from that at either 14 or 18°C (Table 6.1). Among damaged batches of ova there were no significant differences in the mean incidence of holes (ANOVA, $P > 0.05$; Table 6.1).

Table 6.1 Proportion of fish which produced egg batches containing damaged ova, mean (± SEM) incidence of damage in those egg batches and resulting overall prevalence of chorion damage in ova from female Atlantic salmon maintained at 14, 18, or 22°C. Egg batch refers to the ova of a single female. Figures with the same superscript are not significantly different ($P > 0.05$).

	Temperature Regime (°C)		
	14	**18**	**22**
Proportion of Damaged Egg Batches (%)	27.3^{a}	60.9^{b}	38.5^{ab}
Mean Incidence of Damaged Eggs (%)	32.6^{a} ± 5.1	31.9^{a} ± 3.0	38.4^{a} ± 7.8
Overall Prevalence of Egg Damage (%)	8.9 ± 1.4	19.4 ± 1.8	14.8 ± 3.0

There were significant negative correlations between percent survival of ova to the eyed stage and incidence of holes for ova from fish maintained at 14°C ($R = -0.63$, Bartlett χ^2-Statistic = 9.8, d.f. = 1, $P < 0.01$) and at 18°C ($R = -0.47$, Bartlett χ^2-Statistic = 5.2, d.f. = 1, $P < 0.05$) whereas, for ova from fish held at 22°C, there was no significant correlation between the incidence of chorion damage and the survival of eyed ova.

6.4 Discussion

Exposure to elevated temperature during vitellogenesis was associated with significant reductions of both the fertility and the survival of ova from female Atlantic salmon. Mean fertility of ova from fish held at 22°C was less than 70% and subsequent survival to the eyed stage was only ~ 40% whereas, values for both parameters exceeded 80% for fish held at 14 and 18°C. While there have been no reports of directly comparable studies in other salmonids, Chmilevsky (2000) observed arrested gonadal development in rainbow trout held at 22-23°C. In addition, the present results are broadly consistent with those reported by Pankhurst et al. (1996) in rainbow trout, where maintenance at 18 and 21°C for the 3 months prior to ovulation was associated with failure to produce viable ova. Similarly, in a study of coho salmon (*Oncorhynchus kisutch*) from the Great Lakes of North America, Flett et al. (1996) reported highly variable fertility (0 to > 80%) and low survival to hatch (42%) in ova from females which had been exposed to elevated temperatures during the 5 months preceeding spawning. Furthermore, in an examination of reproductive performance in the common wolffish which more closely resembled the design of the present study (albeit at a lower range of temperatures), Tveiten and Johnsen (1999) observed a trend of declining egg survival in association with increasing holding temperature during vitellogenesis. In cyprinid fishes, un-seasonal elevations in temperature can also have detrimental effects such as induction of ovarian atresia (reviewed by Van Der Kraak and Pankhurst, 1997).

On the basis of studies largely conducted on rainbow trout, Bromage et al. (1992, 1993) reported that there was little evidence to suggest that temperature acts as a

proximate cue for the timing of reproduction in salmonids and argued that reproductive phasing was almost totally dependent on photoperiod. However, in female Atlantic salmon, Duncan et al. (2000) suggested that other factors, in addition to photoperiod, may be involved in the control of maturation. Photoperiod manipulation resulted in a 3-week advance in the commencement of ovulation in female salmon whereas, similar artificial photoperiods have been demonstrated to advance ovulation by up to 14 weeks in rainbow trout (Duncan et al., 2000). As a result, it was concluded that a temperature-sensitive mechanism was responsible for the control of maturation in female Atlantic salmon (Duncan et al., 2000). Taranger et al. (2000) also reported that exposure to reduced water temperatures (approximately 5°C below natural) both synchronised and advanced ovulation in Atlantic salmon maintained under natural and accelerated photoperiods. In this regard, the present observation of increased synchrony of ovulation following temperature reduction in fish previously held at 22°C also indicates the involvement of temperature in the control of maturation in female Atlantic salmon and suggests that the magnitude and/or rate of temperature reduction may assist in entraining an endogenous reproductive rhythm in a manner similar to that reported for changes in photoperiod (eg, Porter et al., 2000; Randall et al., 2000).

The fact that the present results contrast with those reported by Tveiten and Johnsen (1999), where maintenance of female wolffish at elevated temperatures during gonadal recrudescence was associated with a 4-5 week delay in ovulation, may indicate differences between the responses of salmon and wolffish to temperature elevation or, alternatively, may simply reflect differences in the timing of temperature reduction. The data presented by Tveiten and Johnsen (1999) indicate that temperature reduction was completed close to the commencement of ovulations in control fish. In contrast, during the present study, temperature reduction was completed ~ 1 month prior to the commencement of ovulations in controls.

In general, the patterns of E_2 and T production observed during the present study agree with those presented earlier (Chapter 2). In mid January, both steroids were recorded at levels of ~2-3 $ng.ml^{-1}$ and levels increased approximately 7 to 10-fold as sampling progressed. In a companion study (Chapter 2) plasma E_2 and T in unmanipulated female Atlantic salmon increased from ~ 2-5 $ng.ml^{-1}$ to 20-25 $ng.ml^{-1}$

over a similar timeframe. As previously discussed (Chapter 2), this pattern also matches that reported in association with ovarian growth in a range of rainbow trout stocks and a Norwegian stock of Atlantic salmon (eg. Scott et al., 1980; Fostier et al., 1983; Scott and Sumpter, 1983; Schulz, 1984; Fostier and Jalabert, 1986; Oppen-Bertsen et al., 1994). Furthermore, the general reduction throughout in E_2 production in fish held at 22°C, along with the significant reduction in both E_2 and T levels at the final sample point in fish held at 18 and 22°C, is consistent with the previous observation of an apparent impairment of ovarian steroidogenesis during the latter stages of vitellogenesis in salmon maintained at 16°C (Chapter 3). This supports the contention that exposure to elevated temperatures may influence vitellogenic as well as maturational processes in Atlantic salmon (Chapter 3), but contrasts with the conclusions of Pankhurst et al. (1996) and Pankhurst and Thomas (1998) who reported inconsistent effects of maintenance at elevated temperatures on ovarian steroidogenesis during vitellogenesis in female rainbow trout. A trend of declining E_2 and T with increasing temperature (9-21°C) was observed in the earlier study (Pankhurst et al., 1996). However, no such trend was noticeable in the later study (Pankhurst and Thomas, 1998) and it was concluded that ovarian steroidogenesis during vitellogenesis was essentially unaffected by elevated temperature across the range 12 – 18°C. It is also possible that rainbow trout share the same response as Atlantic salmon, albeit at a higher temperature than the upper limit used in the trout studies.

The reduction in E_2 and elevation in T observed during the earlier stages of sampling (January to March) in fish held at 22°C in the present study is suggestive of impaired E_2 synthesis. It is well established that production of E_2 by the salmonid ovary occurs in the granulosa cells of the ovarian follicle from androgen substrate produced in the thecal cells (reviewed by Nagahama et al., 1993). The conversion is dependent on the activity of the aromatase cytochrome P450 ($P450_{arom}$) enzyme system. The present observations suggest that the impairment of ovarian steroidogenesis during the early stages of sampling was largely restricted to inhibition of $P450_{arom}$ activity. In contrast, the observation of significant reductions in both E_2 and T in fish from the 18 and 22°C treatments at the final sample point indicates that impairment of ovarian steroidogenesis involved enzymes higher in the synthetic pathway at that stage. In this

context, the response of Atlantic salmon to exposure to elevated temperatures during vitellogenesis appears to differ from that of the Wolffish. Tveiten and Johnsen (2001) also observed a significant reduction in the plasma levels of both E_2 and T in female wolffish associated with exposure to elevated temperature during vitellogenesis, but the reduction in both steroids was evident throughout the 5-month period of exposure to elevated temperature. As a result, Tveiten and Johnsen (2001) concluded that high temperatures acted to inhibit overall steroidogenic capacity in the wolffish.

Vtg levels in fish held at 14 and 18°C tended to be elevated at the commencement of sampling (~ 10 – 15 $mg.ml^{-1}$) but remained relatively constant. In rainbow trout, Tyler et al. (1990) reported Vtg levels of ~ 15 $mg.ml^{-1}$ at a stage corresponding with the start of the present study. However, the absence of a continuing increase in plasma Vtg, concomitant with the observed increases in plasma E_2 and T, is unexpected. In contrast, the pattern of Vtg production observed at 22°C appeared to resemble those reported elsewhere. Plasma Vtg commenced at levels of ~ 5 $mg.ml^{-1}$ and displayed a late rise to levels of ~ 28 $mg.ml^{-1}$ at the final sample. This is in broad agreement with the pattern of Vtg production, either observed directly or inferred from measurements of plasma calcium, in rainbow trout where the plasma titre of Vtg typically increases 1.5 to 2-fold per month, peaking in excess of 30 mg ml^{-1} (reviewed by Bromage and Cumaranatunga, 1988; Tyler and Sumpter, 1996). However, closer comparison with the data presented by Tyler et al. (1990) for rainbow trout shows that, during the corresponding stage of gonadal development in virgin rainbow trout (ie. 1-4 months prior to ovulation), plasma Vtg levels increased from ~ 17 $mg.ml^{-1}$ to 25 $mg.ml^{-1}$. This tends to imply impairment of vitellogenesis at 22°C during January and February in the present study and suggests that the later, rapid elevation in plasma Vtg may reflect a compensatory up-regulation of Vtg production, possibly stimulated by the progressive change in photoperiod.

In this context, the majority of the salmonid literature tends to indicate that increased temperature actually favours rather than impairs Vtg production. For example, Olin and Von Der Decken (1989) observed an increase in serum Vtg from ~ 10 $mg.ml^{-1}$ to ~ 28 $mg.ml^{-1}$ in Atlantic salmon smolts in association with an increase in temperature from 8 to 16°C. Accordingly, these authors calculated a Q_{10} temperature coefficient for Vtg production of 3.71. Similarly, Korsgaard et al. (1986), using serum levels of

alkali-labile phosphorous and calcium as indicators of circulating Vtg, observed a failure to produce Vtg following E_2 treatment in Atlantic salmon post-smolts held at 3°C. In contrast, E_2-treated fish held at 10°C or transferred to 10°C from 3°C exhibited increased serum levels of alkali-labile phosphorous (~ 12 $\mu g.ml^{-1}$) and calcium (~ 4-5 mM) relative to controls (~ 0.7 $\mu g.ml^{-1}$ and ~ 2.8 mM respectively). Similarly, rainbow trout held at 15°C exhibited measurable Vtg production within 24h of E_2 injection, with plasma Vtg peaking at ~ 70 $mg.ml^{-1}$ within 10 days (MacKay and Lazier, 1993). In contrast, in fish held at 9°C plasma Vtg appeared later (~ 72h) and reached a level of only 8.9 $mg.ml^{-1}$ after 10 days. Furthermore, Vtg mRNA was detected first in fish held at 15°C (within 8h) followed by 9°C (~ 24h) and 4°C (~ 48h). However, all the above studies were conducted at temperatures near or below the lowest temperature of the present study. This indicates that, in terms of Vtg production, increasing temperature is stimulatory up to a certain point beyond which it becomes inhibitory. In Atlantic salmon that threshold appears to lie between 18 and 22°C.

Fish eggs take tend not to take up any nutrients from the environment following ovulation. Therefore, all of the contents which determine the quality of the egg must be incorporated into the oocyte during ovarian growth (reviewed by Brooks et al., 1997; Tyler et al., 2000). Accordingly, since uptake of Vtg can account for more than 90% of final oocyte volume (reviewed by Tyler, 1991; Tyler et al., 2000) the significance of impaired vitellogenic growth for gamete quality and subsequent embryo viability cannot be overstated. In rainbow trout, for example, treatments which resulted in a ~ 46% reduction in plasma Vtg levels also resulted in reductions in egg weight and egg volume of ~ 13% and ~ 17% respectively, and were associated with reduced survival of eggs to the eyed stage of development (~ 54% c.f. ~ 96% in controls) (Campbell et al., 1994). During the present study, the size, fertility and survival of ova from fish held at 22°C were also significantly reduced relative to lower temperatures. In addition, significantly reduced levels of Vtg were observed at each of the first three sample points in fish held at 22°C (relative to those at 14°C). In this context, the observation of a weak but statistically significant correlation between egg diameter and the sum of plasma E_2 levels for the first and second samples, as well as the existence of weak but statistically significant correlations between plasma Vtg

levels and plasma E_2 levels at the first and second samples are consistent with the known role of E_2 in stimulating vitellogenesis (reviewed by Momsen and Walsh, 1988; Tyler, 1991; Specker and Sullivan, 1994) and suggest that the reduced performance of ova from fish held at 22°C was associated with the significant reductions in E_2 observed during January and February.

Gonadotropin (GtH)-I is understood to play a role in vitellogenic oocyte growth via its control of ovarian E_2 production (Tyler and Sumpter, 1996). Thus the reduction in plasma E_2 observed in fish held at 22°C during January and February may reflect temperature-related depression of pituitary GtH-I secretion, increased GtH-I clearance at elevated temperature, or the temperature-related failure of GtH-I to stimulate adequate granulosa $P450_{arom}$ activity. Alternatively, the reductions in circulating E_2 and Vtg may represent a stress effect. Carragher et al. (1989) reported a depression of plasma E_2 and Vtg levels in female rainbow trout following artificial elevation of plasma cortisol levels. In later experiments, plasma Vtg levels in rainbow trout were halved following 2 weeks of confinement stress, while implantation of female brown trout with a slow-release cortisol-pellet resulted in reduced plasma E_2 (2 c.f. 7 $ng.ml^{-1}$) and Vtg (6 c.f. 24 $mg.ml^{-1}$)(Pottinger et al., 1991). During the present study, in spite of a general decline as sampling progressed, plasma cortisol levels at all temperatures were relatively high through-out sampling (~ 30-100 $ng.ml^{-1}$). In contrast, chronic confinement stress in rainbow trout and brown trout resulted in plasma cortisol levels of only 6.9-37.5 $ng.ml^{-1}$ whereas, control fish exhibited plasma cortisol levels of 1.8-7.2 $ng.ml^{-1}$ (Campbell et al., 1994). Exposure of rainbow trout to acute low water stress (Pankhurst and Van Der Kraak, 2000) did result in cortisol levels similar to those observed at early sample times in the present study. Following reduction in tank water level to the point where fish were just covered, a rapid (< 1 hour) increase in plasma cortisol from pre-stress levels of 13-22 $ng.ml^{-1}$ to post stress levels of 59-108 $ng.ml^{-1}$ was observed. However, plasma cortisol declined to intermediate levels within 3-6 hours of the restoration of water levels (Pankhurst and Van Der Kraak, 2000). Accordingly, it is likely that the absolute levels of plasma cortisol observed during the present study reflect, at least in part, an acute response to the stress of capture, anaesthesia and blood sampling. Nonetheless, plasma cortisol levels were generally higher at elevated temperatures and there was a weak but

significant negative correlation between plasma cortisol and Vtg levels in early samples, suggesting a temperature treatment-related stress effect on vitellogenesis. However, while there was also a weak but significant positive correlation between plasma Vtg and E_2 levels in early samples, there was no significant relationship between plasma cortisol and plasma E_2. This is not unexpected since the role of cortisol as a direct mediator of stress on reproduction and, in particular, ovarian steroidogenesis has been difficult to demonstrate unequivocally (eg. Pankhurst et al., 1995; Pankhurst, 1998, Leatherland, 1999) and it has only recently become apparent that cortisol-mediated inhibition of reproduction may result from effects at the level of GtH signal-transduction (Pankhurst and Van Der Kraak, 2000).

Crowding stress and cortisol implantation of rainbow trout was also associated with reduced binding of E_2 in hepatic cytosol and nuclear extracts (Pottinger et al., 1991). These results led the authors to contend that in addition to suppression of E_2 secretion, stress-induced elevation of cortisol might impede vitellogenesis by reduction of hepatic E_2 receptor content. More recently, Lethimonier et al., (2000) reported that cortisol implantation of rainbow trout resulted in significant reductions in hepatic E_2 receptor mRNA and Vtg mRNA. Similarly, although no reference was made to stress or cortisol as a possible mediator of temperature effects on vitellogenesis, MacKay and Lazier (1993) proposed that temperature-related differences in Vtg production in Atlantic salmon might reflect changes in the rates of hepatic Vtg mRNA translation and Vtg secretion. However, it remains to be assessed whether temperature-dependent changes in gene expression account for any of the present effects seen in Atlantic salmon.

Pottinger and Pickering (1990) and Pottinger et al. (1991) also reported an elevation of plasma E_2 binding capacity in cortisol-implanted rainbow trout and linked stress-induced disruption of vitellogenesis to plasma binding protein-mediated reduction in the levels of labile E_2. These observations might account for those instances where cortisol implantation of rainbow trout reduced plasma Vtg but not plasma E_2 levels (eg. Pottinger et al., 1991), or where chronic confinement of rainbow trout and brown trout was associated with reduced gamete quality without affecting total plasma E_2 (eg. Campbell et al., 1994). During the present study, any temperature- or stress-related increase in plasma E_2-binding would have served to exacerbate the effect of

the observed temperature-related differences in plasma E_2. Here it is noteworthy that Hobby et al. (2000) actually observed a trend of reduced binding of E_2 by plasma steroid binding protein following confinement stress of rainbow trout. However, it should also be noted that the study of Hobby et al. (2000) focussed on a short-term response (hours) to acute stress whereas, Pottinger and Pickering (1990) and Pottinger et al. (1991) reported on a longer-term (weeks) response to chronic stress.

Vtg uptake has also been shown to be influenced by GtH-I (reviewed by Tyler, 1991; Tyler and Sumpter, 1996), possibly via promotion of the development of patency (ie., intra-follicular channel formation to facilitate oocyte contact with blood-borne proteins; reviewed by Tyler and Sumpter, 1996). Accordingly, Tyler (1991) reported a more than 2-fold increase in the rate of [^{3}H]-Vtg uptake in maturing female rainbow trout following GtH-I injection *in vivo*. GtH-I also stimulated *in vitro* Vtg uptake by trout follicles in a dose-dependent manner. Thus temperature impairment of GtH-I might also account for the temperature-related differences in plasma Vtg levels observed during the later stages of sampling in the present study. Furthermore, the rate of Vtg uptake is also influenced by oocyte surface area (Tyler and Sumpter, 1996) such that any early temperature-related difference in Vtg uptake is likely to be exacerbated over time. In this context, during the present study, the volume of ova from fish held at 14 and 18°C was ~ 15-20% higher than that of ova from fish held at 22°C.

GtH-I and, in turn, E_2 are also responsible for stimulating hepatic synthesis of egg-shell or vitelline envelope (VE) proteins in teleost fish (Hyllner and Haux, 1995). The teleostean VE consists of two layers; a thin outer layer termed the zona pellucida (ZP) and a thicker inner layer termed the zona radiata (ZR; reviewed by Oppen-Berntsen et al., 1994). VE proteins constitute the majority of the ZR and are therefore, also referred to as ZR-proteins (Oppen-Berntsen et al., 1994). E_2, either applied *in vivo* or added to *in vitro* preparations of liver, induced ZR-protein synthesis in a range teleostean groups which represent ~ 62% of all teleost species, including Atlantic salmon, rainbow trout and brown trout (Hyllner and Haux, 1995, Larsson et al., 1995). In Atlantic salmon, plasma levels of ZR-proteins have been demonstrated to exhibit close correlations with plasma levels of both GtH-I and E_2 throughout the annual reproductive cycle (Oppen-Berntsen et al., 1994). Furthermore, in Atlantic

salmon, *in vitro* ZR-protein synthesis has been shown to be more responsive than Vtg synthesis to E_2, while *in vivo* zonagenesis (the hepatic synthesis and ovarian sequestration of ZR-proteins) has been shown to precede vitellogenesis (Celius and Walther, 1998). Against this background, and taking into account the observation by Oppen-Berntsen et al. (1991) that *in vitro* rates of rainbow trout ZR-protein and Vtg synthesis were similar, it is reasonable to expect that the temperature-related differences in E_2 and Vtg observed during the present study might also be reflected in the reduced production of ZR-proteins at high temperature. Consistent with this argument, holes were observed in the chorions of ova sampled during this study, confirming some impairment of VE formation or structural stability. In addition, the fact that those holes were already present at the point of release from the female genital pore confirms the maternal origin of the phenomenon.

The VE and in particular the ZR, which hardens after fertilisation, protects the developing embryo against mechanical damage, desiccation and fluctuating environmental conditions while also exhibiting bactericidal and fungicidal properties (reviewed by Hyllner and Haux, 1995). In consequence, impairment of ZR protein synthesis or ZR assembly is likely to be reflected in lowered egg survival. In the present study, weak but significant correlations were observed between survival and the incidence of shell damage (at least in ova from fish held at 14 and 18°C). However, the possible role of holding temperature as a causal factor is less clear. The number of females which produced damaged ova was highest at 18°C, and there were no significant temperature-related differences in the incidence of chorion damage within affected egg batches. Here, the limitations of the ova sampling should be acknowledged. Only 10-20 ova were sampled from each female and only one quarter of each ovum was examined, while the numbers and sizes of holes were not evaluated and only their presence or absence recorded. Nonetheless, the prevalence of shell damage was lowest and embryo survival highest in ova from fish held at 14°C and the mean incidence of shell damage highest for ova from fish held at 22°C.

Although chorion damage of the type recorded during this study appears not to have been reported previously, the present observations are consistent with the as yet unpublished studies of P.M. Pankhurst and co-workers (Tasmanian Aquaculture and Fisheries Institute), where similar holes were observed in the shells of Atlantic salmon

ova collected from a number of commercial hatcheries. Survivorship was linked to the relative prevalence of holes observed in ova sampled immediately upon release from the genital pore. Examination of the ova during incubation indicated that mortality was most likely as a result of opportunistic bacterial and/or fungal infestation which resulted in either direct mortality of the enclosed embryo, or premature rupture of the chorion and release of an immature larva. Elsewhere, studies on temperature effects on the structure of salmonid egg shells are restricted to attempts to understand the aetiology of soft shell disease (SSD). SSD is characterised by a loss of hydrostatic pressure within the ovum, collapse of the ZR and subsequent mortality of the embryo (Cousins and Jensen, 1994). These changes result from loosening of the plugs which seal the pore canals of the ZR and colonisation by opportunistic bacterial pathogens which occur as a consequence of egg incubation at elevated temperatures (> 10°C) (Cousins and Jensen, 1994). Thus, in contrast to the present observations, SSD can be regarded as a post-fertilisation phenomenon.

In summary, maintenance of female Atlantic salmon at a water temperature of 22°C during vitellogenesis was associated with a general reduction in plasma E_2 levels and an early reduction in plasma Vtg levels relative to those observed in fish held at 14 and 18°C. A resultant reduction in maternal investment was confirmed by a significant reduction in oocyte diameter in ova from fish held at 22°C while a concomitant increase in the incidence of previously undescribed chorion damage indicated impairment of zonagenesis as well as vitellogenesis. Elevated temperature may have acted directly at the level of the reproductive endocrine cascade or indirectly via the stress axis. The net effect was a significant reduction in the viability of ova from fish exposed to high temperatures during vitellogenesis.

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CHAPTER 7

GENERAL DISCUSSION

7 GENERAL DISCUSSION

7.1 Background

The present studies were undertaken in order to better characterise the reproductive development of female Atlantic salmon cultured in Tasmania, to examine how that development was influenced by elevation of environmental temperature, to identify potential remedial strategies and thereby, contribute to the knowledge base in relation to the environmental management and endocrine manipulation of salmonids. In this regard, the literature relating to possible negative effects of temperature on reproductive processes in fish is relatively sparse, leading Van Der Kraak and Pankhurst (1997) to conclude there is inadequate information upon which to base predictions about the likely effects of changes in environmental temperature on reproduction in fish. Nevertheless, in salmonids, previous studies in Atlantic salmon (Taranger and Hansen, 1993), Arctic charr (*Salvelinus alpinus*) (Gillet, 1991) and rainbow trout (*Oncorhynchus mykiss*) (Pankhurst et al., 1996; Pankhurst and Thomas, 1998) had indicated that temperature effects on reproduction were largely restricted to impairment of final oocyte maturation (FOM) and ovulation but, as the only endocrine data available were derived from rainbow trout (Pankhurst et al., 1996; Pankhurst and Thomas, 1998), the endocrine correlates of temperature-impaired reproduction in Atlantic salmon were undescribed.

7.2 The present studies

7.2.i Reproductive development in female Atlantic salmon in Tasmania

The early phase of the present studies (Chapter 2 and 3) essentially confirmed that the growth and gonadal development of Tasmanian female Atlantic salmon was consistent with maturation as an autumn spawning stock. In agreement with the general pattern of reproductive development reported elsewhere in other salmonids (eg. Bromage and Cumaranatunga, 1988; Tyler et al., 1990), gonado-somatic index (GSI), oocyte size/volume, and plasma 17β-estradiol (E_2) and testosterone (T) levels increased by orders of magnitude between Spring (October) and early Autumn (March). Similarly, during the periovulatory period (Chapter 3), the pattern of ovarian sterodogenesis in fish held at low temperatures (6°C) was characterised by pre-ovulatory declines in

plasma E_2 levels and peaks in plasma T and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) levels which agreed closely with published steroid profiles for other salmonids (eg. Fostier et al., 1983; Van Der Kraak et al., 1984; Fostier and Jalabert, 1986) and other stocks of Atlantic salmon (eg. Crim et al., 1986) (Figure 7.1 cf Chapter 1, Figure 1.12).

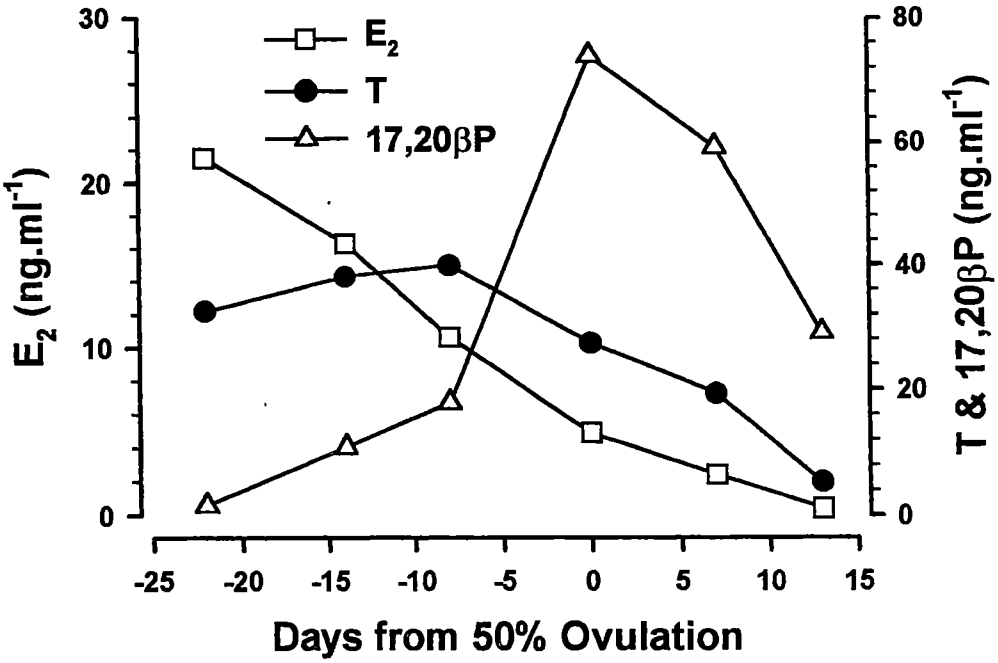

Figure 7.1 Periovulatory plasma steroid hormone levels in Tasmanian female Atlantic salmon displaying characteristic preovulatory declines in 17β-estradiol (E_2) and peaks in testosterone (T) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP). Fish were maintained at 6°C. Error bars are omitted for clarity.

7.2.ii Temperature effects during maturation

The present studies (Chapter 3 and 4) agree with previous reports which demonstrated the inhibitory effects of maintenance at elevated temperatures on ovulation and egg survival in Atlantic salmon and other salmonids (Gillet, 1991; Taranger and Hansen, 1993; Pankhurst et al., 1996; Pankhurst and Thomas, 1998). Elsewhere in Atlantic salmon the incidence of ovulation in fish held at 13-14°C was reduced relative to that observed at lower temperatures (10°C) (Taranger and Hansen, 1993). During the present studies, ovulation was inhibited in fish held at

16°C and delayed in fish held at 11°C. Thus, it appears that, in Atlantic salmon, a progressive decline in ovulatory ability occurs across an ~ 5°C temperature range between 10 and 16°C. In rainbow trout, the corresponding range is ~ 12-18°C (Pankhurst and Thomas, 1998) indicating that the response of Atlantic salmon is similar to that of rainbow trout albeit at a lower absolute temperature range.

In salmonids, oocyte maturation and ovulation are largely stimulated by maturational gonadotropin (GtH-II) (Nagahama, 1997; Goetz and Garczynski, 1997). Importantly, the maturation-inducing activity of GtH-II is mediated by the steroid hormone 17,20βP, production of which follows a two cell type model where the precursor steroid 17α-hydroxyprogesterone (17P) is produced in the thecal layer of the ovarian follicle and converted to 17,20βP in the granulosa layer by the enzyme 20β-hydroxysteroid dehydrogenase (20β-HSD) (Figure 7.2a) (reviewed by Nagahama, 1997). However, 17P must first be generated from cholesterol via pregnenolone and progesterone by the enzyme systems cholesterol side-chain cleavage cytochrome P-450 ($P450_{SCC}$), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase/17-20 lyase cytochrome P-450 ($P450_{C17}$) (Figure 7.2a) (reviewed by Nagahama, 1997).

The temperature sensitivity of the above processes has received limited study and endocrine data are limited to those produced by Pankhurst et al., (1996) and Pankhurst and Thomas (1998) in the rainbow trout. Both *in vitro* and *in vivo* studies indicated that production of 17,20βP was impaired at elevated temperatures (18°C) (Pankhurst and Thomas, 1998).

Initial studies conducted during the periovulatory period (Chapter 3 and 4) demonstrated that female Atlantic salmon maintained at elevated temperatures (16°C) were also incapable of producing 17,20βP, even after luteinizing hormone releasing hormone analogue (LHRHa) administration. Together with the above observations by Pankhurst and Thomas (1998), these results pointed to the conclusion that inadequate GtH-II activity and/or a lack of 20β-HSD were responsible for the observed failure of heat-exposed fish to ovulate or to produce significant quantities of 17,20βP. However, treatment with 17P (Chapter 5) resulted in significant 17,20βP production

in fish held at 16°C, suggesting substrate limitation for ovarian steroidogenesis rather than a lack of 20β-HSD accounted for the absence of 17,20βP in untreated fish (Figure 7.2a). This may have resulted from heat-shock-related inhibition of cholesterol metabolism (Chapter 5) or perhaps inadequate GtH-II stimulation of the enzyme systems associated with the thecal steroid cascade. The fact that 17P-treated fish still did not ovulate, in spite of marked 17,20βP production, tends to imply the latter since GtH-II probably plays a role in stimulation of ovulation as well as FOM (Figure 7.2b). Maturational processes prior to ovulation but downstream of 17,20βP production could also have been affected (Figure 7.2b).

Thus, while the detrimental effects of increased temperature can be exercised at all levels of the endocrine cascade (reviewed by Van Der Kraak and Pankhurst 1997), it appears that, contrary to the conclusions of Pankhurst and Thomas (1998) for rainbow trout, 20β-HSD in Atlantic salmon is unaffected by temperature elevation during the periovulatory period (Figure 7.2b). Instead, substrate limitation of 17,20βP production was apparent and probably resulted from temperature impairment of GtH metabolism (Figure 7.2b). It remains unclear whether high temperatures alter pituitary GtH secretion, clearance of GtH from the plasma, GtH receptor interactions or subsequent GtH signal transduction (Figure 7.2b). However, Pankhurst et al. (1996) saw no significant differences in plasma levels of GtH in rainbow trout maintained at a range of temperatures, while follicles from heat-exposed rainbow trout failed to produce 17,20βP in response to *in vitro* stimulation with human chorionic gonadotropin (hcG). Therefore, the latter two factors would appear to be the most likely candidates.

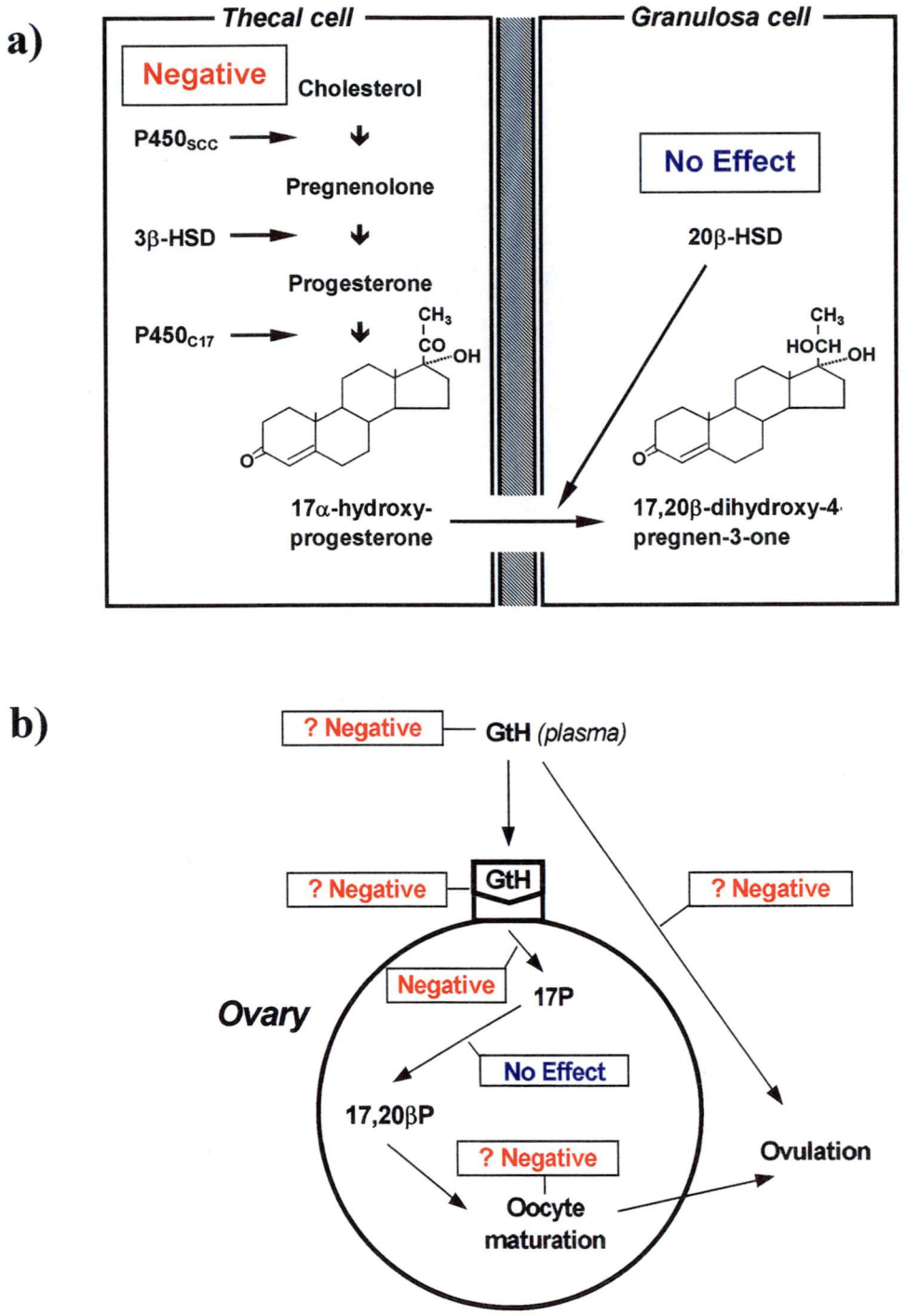

Figure 7.2 Summary of the reproductive endocrine system of female Atlantic salmon (*Salmo salar*) during the periovulatory period showing the effects of elevated temperature on **a)** follicular steroidogenesis and **b)** GtH mediated processes. Possible, but as yet unconfirmed, effects are denoted by a question mark (adapted from Nagahama, 1997 and Pankhurst and Van Der Kraak, 1997).

7.2.iii Temperature effects during vitellogenesis

The present studies (Chapter 6) demonstrate that, in Atlantic salmon, vitellogenesis (the phase of rapid oocyte growth) is unimpaired at temperatures as high as 18°C. This is in agreement with other studies in Atlantic salmon and rainbow trout, conducted in the temperature range 3 - 16°C, where increasing temperature actually has a stimulatory effect on vitellogenesis (eg. Korsgaard et al., 1986; Olin and Von Der Decken, 1989; MacKay and Lazier, 1993). However, poor survival and reduced size of ova from fish held at 22°C during vitellogenesis (Chapter 6) indicates that, in Atlantic salmon, the otherwise stimulatory effect of elevated temperature becomes a deleterious effect at some point between 18 and 22°C.

During vitellogenesis, hepatic Vtg production and ovarian Vtg uptake are stimulated by the follicle-stimulating form of GtH (GtH-I) (reviewed by Tyler and Sumpter, 1996; Nagahama, 1997). The stimulatory effect of GtH-I on Vtg synthesis is mediated by the steroid hormone E_2. E_2 production also follows a two cell type model where T is produced in the thecal layer of the ovarian follicle as a precursor for conversion to E_2 in the granulosa layer by the aromatase cytochrome P450 ($P450_{arom}$) enzyme system (Figure 7.3a) (reviewed by Nagahama, 1997). First, however, T must be synthesised through an extension of the thecal steroidogenic cascade described earlier. Accordingly, 17P is converted to androstenedione and then T by the enzyme systems $P450_{C17}$ and 17β-hydroxysteroid-dehydrogenase (17β-HSD) (Figure 7.3a) (reviewed by Nagahama, 1997). Following release into the circulation, E_2 interacts with hepatic estrogen receptors (ER) to stimulate Vtg synthesis (reviewed by Tyler and Sumpter, 1996). Vtg is subsequently secreted into the circulation and delivered to the ovary where GtH-I may play a role in stimulating its receptor-mediated uptake (reviewed by Tyler at al., 2000).

Although Chmilevsky (2000) reported that gonadal development was arrested in rainbow trout held at 22-23°C, the effect of chronically elevated temperature on vitellogenic endocrine processes has not been reported previously. On the basis of the present studies, the mechanisms responsible for the apparent temperature-related impairment of vitellogenesis remain unclear and it is possible that different mechanisms operated at different phases of oogenesis. In early samples, plasma levels

of E_2 were reduced in fish held at high temperature (22°C) whereas, T levels were elevated (Chapter 6). This suggests impairment of $P450_{arom}$ at the lower end of the endocrine cascade (Figure 7.3a). However, later in vitellogenesis, both E_2 and T levels were depressed, suggesting impairment of processes at a higher level in the endocrine cascade (Figure 7.3a).

Temperature may have acted directly on ovarian steroidogenesis or indirectly via the stress axis and its primary mediator cortisol. In this context, it is noteworthy that the greatest temperature-related elevation in plasma cortisol levels was observed during the early stages of sampling. As outlined above, plasma levels of E_2 and T were differentially affected during that phase of development. However, Pankhurst and Van Der Kraak (2000) recently reported that cortisol's action appears to be manifested relatively high in the endocrine cascade (possibly at the level of GtH signal transduction) such that the effects of stress are mainly reflected by reduced plasma levels of T. Thus, while the depression of ovarian steroidogenesis observed during later samples may have reflected a stress effect, it is more likely that the depression of E_2 observed in early samples was the result of a direct temperature effect. Alternatively, the observations by Pankhurst and Van Der Kraak (2000) of a greater effect of cortisol on T than on E_2 may represent yet another example of the confusing nature of the stress – reproduction interaction where the effects of exposure to stress or cortisol administration on gonadal steroidogenesis have been difficult to demonstrate with consistency (eg. Pankhurst et al., 1995, Pankhurst, 1998, Leatherland, 1999).

Temperature and/or cortisol may also have impaired other aspects of GtH-I metabolism (Figure 7.3b) but, irrespective of the mechanisms responsible, plasma Vtg levels were reduced in early samples from fish held at 22°C, confirming temperature impairment of vitellogenin synthesis (Figure 7.3b).

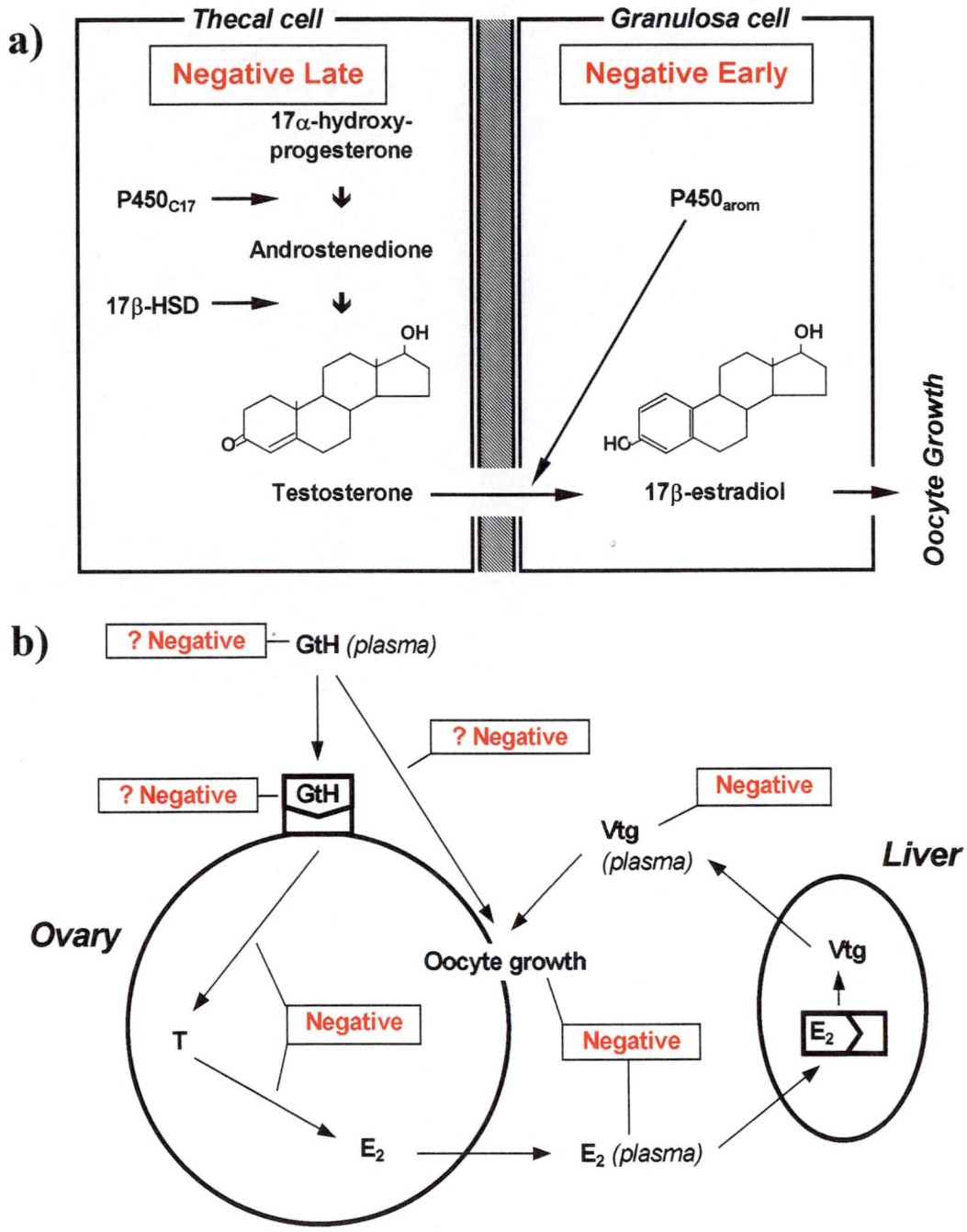

Figure 7.3 Summary of the reproductive endocrine system of female Atlantic salmon (*Salmo salar*) during vitellogenesis showing the effects (either direct or cortisol-mediated) of chronic temperature elevation on **a)** follicular steroidogenesis and **b)** the liver/gonad axis. Possible, but as yet unconfirmed, effects are denoted by a question mark (adapted from Nagahama, 1997 and Pankhurst and Van Der Kraak, 1997).

7.3 Implications for Industry

The present characterisation of reproductive development in Tasmanian female Atlantic salmon (Chapter 2 and 3) is of significance in that it provides the basis for the subsequent experiments as well as a reference data set for other work such as industry's ongoing efforts to control precocious sexual maturation in stock being grown-out for harvest. As previously discussed (Chapter 1), the Tasmanian Atlantic salmon farming industry is uniquely troubled by this phenomenon, but attempts to control it through photo-manipulation of sea-cage reared stock have been uncoordinated and unsuccessful. However, an industry-based programme of collaborative research will soon commence, and the present data will be used to assess the relative effectiveness of different control strategies and to evaluate the usefulness of tools developed for non-destructive assessment of reproductive condition (eg. measurement of mucus steroid levels).

As previously discussed (Chapter 1), natural summer and early autumn elevation of water temperature is already recognised as a potential constraining factor for forward phase-shifting of spawning and egg production in cultured Atlantic salmon (eg. Duncan et al., 2000; Taranger et al., 2000). The apparent failure of LHRHa treatments to stimulate ovulation in fish held at elevated temperatures (Chapter 4) indicates that LHRHa treatment alone is likely to be of only limited value in overcoming temperature-related constraints on off-season reproduction. However, along with the observations of Pankhurst and Thomas (1998), two experiments (Chapter 3 and 4) highlighted the potential for a short-term reduction in temperature to restore ovulation in heat-exposed fish.

Subsequently, it was confirmed (Chapter 5) that short-term temperature ramp-down could indeed restore ovulation in heat-exposed female Atlantic salmon but that, unless LHRHa treatment was combined with temperature reduction, subsequent egg fertility was significantly impaired. In this regard, Duncan et al. (2000) demonstrated that, in Atlantic salmon, the maximum advance in the timing of ovulation which could be achieved through photoperiod manipulation alone was ~ 3 weeks. Meanwhile, Taranger et al. (2000) demonstrated that an advance of at least 8 weeks could be achieved with concomitant temperature reduction to below 8°C. The present studies

indicate that in animals exposed to a simulated natural photoperiod, combined LHRHa treatment (25 $\mu g.kg^{-1}$) and modest temperature reduction (to 11°C) are capable of yielding 100% ovulation within 14 days. If such a response were to be maintained in photo-manipulated stock, advances in the timing of ovulation similar to those achieved by Taranger et al. (2000) might be achieved, if not exceeded, with a reduced requirement for water-cooling. This would have particular significance for Tasmanian Atlantic salmon farming industry where the potential value of further extending 0+ smolt production through manipulation of broodstock and advancing the timing of spawning has been estimated to be AUS $ 8 – 16 million per annum (confidential industry estimate prepared for the Co-operative Research Centre for Aquaculture in 1998).

Following maintenance of female Atlantic salmon at elevated water temperatures during vitellogenesis a reduction in egg diameter reflected a temperature-related reduction in maternal investment and a concomitant increase in the incidence of malformation of, or damage to, the egg chorion. This indicated that zonagenesis had also been impaired. These observations and the associated marked decline in egg viability have particular significance for smolt production in Tasmania. Since the upper thermal limits for successful husbandry of Atlantic salmon can be approached in Tasmania and the limits for successful reproduction can be exceeded, the question of temperature-related impairment of gamete quality may have to be addressed periodically as the natural cycle of climatic variation (eg. El Niño) results in warm years. Continuous management of broodstocks may also be required if sustained warming occurs in association with global climate change. From a global perspective, while the problems of climate change will come first to Tasmania, there is potential for the entire production of farmed salmon to be affected. In this regard, the present studies suggest that during summer, maintenance of Atlantic salmon broodstock at temperatures of 18°C or lower should be sufficient to ensure the subsequent production of high-quality gametes. Conversely, prolonged exposure of broodstock to temperatures which exceed 18°C can be expected to significantly impair gamete quality and thereby jeopardise the continuing capacity of the propagation sector of the Tasmanian Atlantic salmon farming industry to produce smolts for the AUS $ 120 million per annum grow-out sector.

The present studies also have relevance for wild salmonids and for conservation biology. As previously discussed, in view of their narrow thermal tolerance range, salmonids are considered to be stenothermal (Wedemeyer, 1996) and are known to exhibit strong behavioural thermoregulation (eg. Welch et al., 1995). As a consequence, it has been concluded that future changes in ocean temperature could have a profound effect on the dynamics of salmon production (Welch et al., 1995). Furthermore, changing water- and land-use practices have the potential to alter the thermal regimes of inland waters in the short-term. Snucins and Gunn (1995) showed that Canadian populations of lake trout (*Salvelinus namaycush*) actively seek-out and occupy areas of cold groundwater discharge in otherwise warm bodies of water. Accordingly, these authors argued that such potentially critical habitats should be protected. Failure to take account of such observations and advice could result in the exposure of stocks to thermal regimes which impair and/or delay reproduction in a manner consistent with the present results.

7.4 Future studies

Not unexpectedly, the present studies leave many questions unanswered and there is therefore, considerable scope for further study. At the most basic level, analysis of Vtg levels in stored plasma from earlier experiments (principally Chapter 2) will complete the characterisation of reproductive development in female Atlantic salmon in Tasmania and comparison with data from later studies (Chapter 6) may help to clarify whether there was any impairment of vitellogenesis during the earlier study.

In spite of strong circumstantial evidence for an effect of elevated temperature on GtH metabolism (Chapter 4 and 5), it has only been possible to speculate on the nature of any temperature effect on GtH. Accordingly, analysis of GtH in stored plasma samples should assist to clarify how elevated temperature affects FOM and ovulation in Atlantic salmon. Absence of temperature-related differences in plasma levels of GtH would agree with the observations of Pankhurst et al. (1996) and point to effects at the level of GtH receptor interaction and/or subsequent signal transduction. In contrast, detection of temperature-related differences in plasma GtH

levels would tend to support effects on pituitary GtH synthesis and/or the longevity of GtH in the plasma.

In view of the apparent success of combined temperature ramp-down and LHRHa treatment in restoring ovulation in heat-exposed female Atlantic salmon, and considering the potential consequences for gamete viability, understanding how exposure to elevated temperatures affects oogenesis (particularly vitellogenesis) becomes a critical strategic issue for industry. As previously discussed, the mechanisms by which elevated temperature impairs reproduction are unclear but may include increased GtH clearance from the plasma and subsequent reduction in ovarian ligand-receptor interaction (see Van Der Kraak and Pankhurst, 1997). Similarly, the rate at which free steroid is inactivated via conjugation is increased at high temperatures (reviewed by Van Der Kraak and Pankhurst, 1997). The processes involved in signal transduction are also understood to be temperature sensitive (Van Der Kraak and Wade, 1994). E_2 –ER interaction may be temperature labile and Mackay and Lazier (1993) have reported amplification of hepatic ER content and Vtg gene expression in response to increasing temperature, while induction of heat-shock proteins (produced to restore tertiary protein structure following thermal insult) may also affect aspects of gene activation.

Whereas the present studies were conducted at the whole animal level, the above considerations demonstrate the necessity of conducting future studies at the tissue and/or molecular levels. Accordingly, in order to determine where temperature-related blockade of the reproductive endocrine cascade occurs and to indicate additional remedial strategies for application *in vivo*, a programme of *in vitro* analysis is proposed.

Using previously developed protocols (eg. Haddy and Pankhurst, 1998), temperature effects on ovarian GtH-receptor binding, cAMP activation of intracellular messengers, and the activity of steroid-converting enzymes may be assessed following measurement of *in vitro* steroid production by ovarian follicles at a range of GtH and steroid precursor concentrations, and intracellular cAMP levels. Similarly, existing techniques for preparation of hepatic cytosol (Pottinger, 1986) and radioreceptor

measurement of steroid binding protein characteristics (Hobby et al., 2000) may be used to assess hepatic ER affinity and binding capacity.

Vtg mRNA expression may be studied by *in situ* hybridisation (Gagne and Blaise, 2000) and Northern blotting (Mazurais et al., 2000) of hepatic tissue (with and without *in vitro* E_2 stimulation) using cDNA probes synthesised from the known rainbow trout cDNA Vtg sequence (Goulas et al., 1996) or probes derived from cloned Atlantic salmon cDNA. Furthermore, the Vtg ELISA described during the present studies (Chapter6) may be employed to measure Vtg in E_2-treated hepatic slices or cultured hepatocytes, and the localisation of Vtg secretion and expression of Vtg mRNA transcripts (from *in situ* hybridisation) may be compared using protein immunohistochemistry.

7.5 Concluding remarks

Reviewers of the literature relating to fish reproduction invariably preface, or conclude, their articles with comments which emphasise the undeniable significance to any aquaculture industry of a regular and reliable supply of high-quality juveniles as a fundamental pre-requisite for the development of a vibrant grow-out sector (eg. Bromage et al., 1992; Pankhurst and Van Der Kraak, 1997).

In this context, the potentially deleterious effect of elevated water temperature on gamete quality is acknowledged as one of the most significant medium- to long-term threats to the continued success of SALTAS' operations as well as the sustainability of the Tasmanian Atlantic salmon farming industry in general. As a consequence, understanding thermal inhibition of reproduction in salmon is of immense importance. To this end, while a great deal of further work is required in order to clarify the mechanisms involved, the present studies provide guidelines for the thermal management of Atlantic salmon broodstock during vitellogenesis, and for the endocrine manipulation of maturing female Atlantic salmon in order to restore ovulation during periods of exposure to elevated environmental temperature.

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